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Anti-sense RNA efficiently inhibits formation of the 10 kd polypeptide of photosystem II in transgenic potato plants: analysis of the role of the 10 kd protein

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A chimeric gene encoding an anti-sense RNA of the 10 kd protein of the water-splitting apparatus of photosystem II of higher plants under the control of the CaMV 35S promoter was introduced into potato using *Agrobacterium* based vectors. The expression of the anti-sense RNA led to a significant reduction of the amounts of the 10 kd protein and RNA in a number of trangenic plants. In three out of 36 plants tested, the level of the 10 kd protein was only up to 1–3% compared with the wild-type control. The drastic reduction of the 10 kd protein did not influence the accumulation of other photosystem II associated polypeptides at both the RNA and protein level. Furthermore no phenotypic differences were observed between potato plants expressing wild-type and drastically reduced levels of the 10 kd protein with respect to growth rate, habitus or ultrastructure of the chloroplasts. Measurements of the relaxation of the flash-induced enhancement in the fluorescence quantum yield as determined in intact leaves and the rates and characteristic oscillation pattern of O_2 evolution as determined in isolated thylakoid samples however, show that the elimination of the 10 kd protein on the one hand retards reoxidation of Q_A^- and on the other hand introduces a general disorder into the PSII complex.

Key words: anti-sense RNA/photosystem II/10 kd protein/ transgenic plants

Introduction

Photosynthesis represents a process of fundamental importance with respect to the development and maintenance of all organisms. Durig recent years the combined application of both molecular biology and biochemical/biophysical methods has considerably improved both the understanding of mechanisms underlying the process of photosynthesis and structural features of the complex involved (Glazer and Melis, 1987; Andréasson and Vänngard, 1988; Rochaix and Erickson, 1988). Probably the best understood photosynthetically active complex is the photosynthetic reaction centre of the purple bacterium *Rhodopseudomonas viridis* where X-ray crystallographic analysis has led to a complete unravelling of its structure (Deisenhofer *et al.*, 1984, 1985).

It is obvious that the results of these experiments are of ultimate importance also for the understanding of the structure of the reaction centre of photosystem II of higher plants. In contrast to the reaction centre of purple bacteria, however, photosystem II is capable of light-induced oxidation of water, thus being the major source of all oxygen found on earth. In higher plants, photosystem II is formed by a large number of different polypeptides the majority of which can be assigned to three functional domains, the light-harvesting system, the photochemical reaction centre and the water-splitting activity. Some of these polypeptides are encoded by the plastid genome and others are the products of nuclear genes (Herrmann *et al.*, 1985; Rochaix and Erickson, 1988).

While the location of the functional redox groups of the photosystem II reaction centre within a heterodimer of polypeptides D1 and D2 is now widely accepted (Trebst, 1986; Deisenhofer and Michel, 1989), the structural basis of the water-splitting complex still needs to be unravelled (Renger, 1987; Homann, 1988; Brudvig *et al.*, 1989; Rutherford, 1989). Four polypeptides of 33, 23, 16 and 10 kd located in the thylakoid lumen are closely associated with this activity (Jansson *et al.*, 1979; Åkerlund *et al.*, 1982; Kuwabara and Murata, 1982; Yamamoto *et al.*, 1983; Ljungberg *et al.*, 1986; Miyao and Murata, 1989). However, none of them carries the catalytic manganese cluster, they are rather of structural and regulatory importance for the functional integrity of the water-splitting complex (Homann, 1988; Brudvig *et al.*, 1989; Rutherford, 1989). With regard to the central role these polypeptides play in the process of oxygen evolution, a more complete understanding of their function would be highly desirable. In the past, reconstitution experiments (Åkerlund *et al.*, 1982; Ono and Inoue, 1984) and mutational analysis (Mayfield *et al.*, 1987a,b) have mainly been used to exploit the contribution of the different polypeptides with respect to oxygen evolution. These approaches have led to the conception that the 33 kd polypeptide is necessary to preserve the manganese in its active site (Ohio and Inoue, 1983, 1984); it is thus an obligatory requirement for photosystem II activity *in vivo* (Mayfield *et al.*, 1987a). On the other side, the 23 kd polypeptide is to some extent dispensable. A *Chlamydomonas* mutant lacking this protein still maintains the capacity of light-induced oxygen evolution, although at very reduced levels (Mayfield *et al.*, 1987b). As inferred from biochemical analysis, the function of the 23 kd protein is mainly concerned with the binding of Ca^{2+} and chloride, two essential cofactors for photosystem II activity (for a review: Homann, 1988, Rutherford, 1989).

In contrast to what is known about the roles of the 33 kd and 23 kd polypeptides, only limited information is available concerning the roles of the 16 and 10 kd proteins. No mutants have been isolated yet to elucidate their functions

in vivo. Ljungberg and coworkers (Ljungberg *et al.*, 1984, 1986) have obtained some evidence indicating that the 10 kd protein serves a structural role in the water-splitting complex by providing binding sites for the 23 kd polypeptides. However, no clear-cut conclusions could be drawn from these reconstitution experiments.

In order to gain more insight into the function of the 10 kd protein and to circumvent the problems inherent in *in vitro* experiments we decided to use an *in vivo* approach using reversed genetics with the aim of suppressing the expression of the 10 kd protein in intact plants. To this end we introduced chimeric genes into transgenic potato plants expressing an anti-sense RNA with respect to the 10 kd protein.

Here we describe the results of the analysis of potato plants expressing the wild-type level of the 10 kd protein in comparison with transgenic potato displaying only 1–3% of the wild-type level with respect to various physiological, biochemical and biophysical parameters.

Results

Construction of the 10 kd anti-sense gene and its integration into the potato genome

A full length cDNA clone of the 10 kd transcript (Eckes *et al.*, 1985) was fused in reverse orientation to the strong promoter of the CaMV 35S transcript (position −526 to +4 relative to the transcription start site), followed by the polyadenylation signal of the octopine synthase gene derived from the T-DNA of the octopine plasmid pTiACH 5 (Figure 1).

This construct was inserted into the binary vector Bin 19 (Bevan,1984) and *Agrobacteria* transformed with this construct were used for transforming potato plants via the leaf disc technique (Rocha-Sosa *et al.*, 1989). Regenerated plants were tested via DNA blot hybridization experiments for the correct integration of the anti-sense gene (data not shown) and only those plants containing non-rearranged copies of the construct were used in further experiments.

Some transgenic plants contain a dramatically reduced level of the 10 kd protein

Thirty-six transgenic potato plants containing the anti-sense construct were grown in the greenhouse. In between these plants no differences concerning growth rates and habitus were observed (see below). In order to determine whether or not any of these transgenic plants would be affected with respect to the expression level of the 10 kd protein, all 36 plants were screened by Western blot analysis. Within the population analysed the amount of the 10 kd protein varied by a factor of ~100. In six out of the 36 plants tested the amount of this protein was below the detection limit imposed by this kind of analysis (data not shown).

The reduction of the 10 kd protein is seemingly unrelated to the expression level of the anti-sense RNA

Twelve plants displaying drastic differences with respect to the amount of 10 kd protein present were chosen for a more detailed analysis at both RNA and protein levels. In order to estimate the amount of transcript encoding the 10 kd protein at the steady state RNA level, total RNA extracted from leaves was probed with a strand specific anti-sens RNA probe generated by the SP6/T7 transcription system (Figure 2A) in a RNA blot experiment.

Fig. 1. Structure of the chimeric gene expressing an anti-sense RNA of the 10 kd protein encoding cDNA. The promoter of the CaMV 35S RNA (position −526 to +4 relative to the transcription start site) was fused in front of a full length cDNA clone of the 10 kd a transcript (Eckes *et al.*, 1985) and provided with the poly A site of the octopine synthase gene of the Ti-plasmid pTiACH 5. The 5′ to 3′ direction of the promoter is indicated by a filled triangle, the (original) 5′ to 3′ direction of the cDNA clone is indicated by an open triangle.

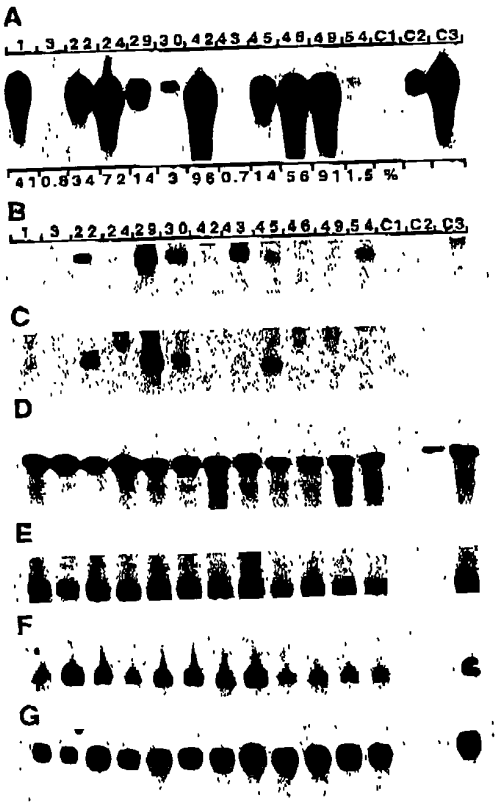

Fig. 2. Northern analysis of total RNA isolated from leaves (A, B, D–G) or roots (C) of different transgenic and control potato plants. After isolation total RNA (50 μg each except lanes C1, C2, C3) was subjected to gel electrophoretic separation using formaldehyde gels (cf. Materials and methods), blotted on nylon membranes and subjected to hybridization using different molecular probes. The following probes were used: A: an RNA probe encoding the anti-sense RNA of the 10 kd protein; B,C: an RNA probe encoding the sense RNA of the 10 kd protein; D: a nick-translated DNA probe encoding the plastid gene psbA; E: a nick-translated DNA probe encoding the plastid gene psbB; F: a nick-translated cDNA probe encoding the nuclear 33 kd polypeptide; G: a nick-translated cDNA probe encoding the nuclear small subunit of the ribulose bisphosphate carboxylase gene. The numbers 1, 3, 22, 24, 29, 30, 42, 43, 45, 46, 49, 54 on top of panel A identify different independently transformed potato plants harbouring the construct encoding the anti-sense RNA to the 10 kd protein (cf. Figure 1). Lanes C1, C2 and C3 contain 0.5, 5 and 50 μg of total RNA isolated from leaves of a nontransformed control potato plant. The numbers given below the lanes in panel A indicate the relative amount of the 10 kd transcript in percentage as compared with the level observed in a wild-type plant.

The relativ transcript levels as determined by scanning the autoradiographs with a laser scanner are given in Figure 2A. Plant numbers 3, 30, 43 and 54 contain the lowest amount of 10 kd encoding RNA amounting to −1−5% of the wild-type level whereas the level of this RNA in the plant nos 42 and 49 does not significantly differ from that found in wild-type plants (cf Figure 2A).

The same blots were also used to determine the corresponding amount of the anti-sense RNA transcribed from the transferred chimeric gene by using sense RNA as probe (Figure 2B). In addition. as the estimation of the amount of anti-sense RNA present in leaves could be misleading due to the presence of sense RNA total root RNA which is devoid of sense RNA was included in this analysis (Figure 2C). In some transformants the relative amount of detectable anti-sense RNA was similar in leaves and roots (Figure 2B and C: plant numbers 1, 22, 29,30), whereas in others the relative amount varies between leaves and roots (Figures 2B and C: plant numbers 43,45,54). It is important to note however that there is no obvious correlation between the amount of anti-sense RNA detectable and the suppression of the 10 kd transcript levels (compare Figures 2A-C).

Level of steady state RNA of other nuclear and plastid genes involved in photosynthesis

As outlined in the Introduction, photosystem II is composed of a large number of polypeptides encoded either by the nuclear or the plastid genome. The expression of both sets of genes therefore has to be co-ordinated by signals exchanged between the chloroplasts and the nucleus. Within this respect one might expect that the reduction of the 10 kd protein and its transcript levels would have an influence on the accumulation of either other nuclear and plastid encoded photosystem II transcripts or on the transcript levels of other nuclear genes encoding chloroplast-located proteins such as the genes encoding the small subunit of the ribulose bisphosphate carboxylase. The plastid genes *psbA* (Figure 2D), *psbB* (Figure 2E), as well as a cDNA from the nuclear encoded 33 kd polypeptide (which is a component of the oxygen-evolving complex of photosystem II) (Figure 2F) and a cDNA encoding the small subunit of the ribulose bisphosphate carboxylase (Figure 2G) were used as probes. (Note that in Figure 2E, only the band representing the mature RNA of the *psbB* hybridizing transcripts with a size of 1800 nucleotides is shown for this comparison).

A comparison of the transcript levels of these genes in different transgenic potato plants and wild-type plants indicates that they are seemingly not influenced by the expression level of the 10 kd protein (compare Figures 2A−G).

Steady state level of photosystem II polypeptides

In order to answer the question of whether the 10 kd polypeptide is an essential structural component involved in the assembly and/or stabilization of other photosystem II polypeptides the amount of steady state protein of some photosynthetic polypeptides was analysed in transgenic potato plants harbouring the 10 kd anti-sense contruct. To this end extracts from young and old leaves were analysed by Western blot experiments. As shown in Figure 3A, the level of the 10 kd protein closely follows the steady state RNA levels (compare Figures 2A and 3A). This result was obtained by using protein extracts from both young leaves

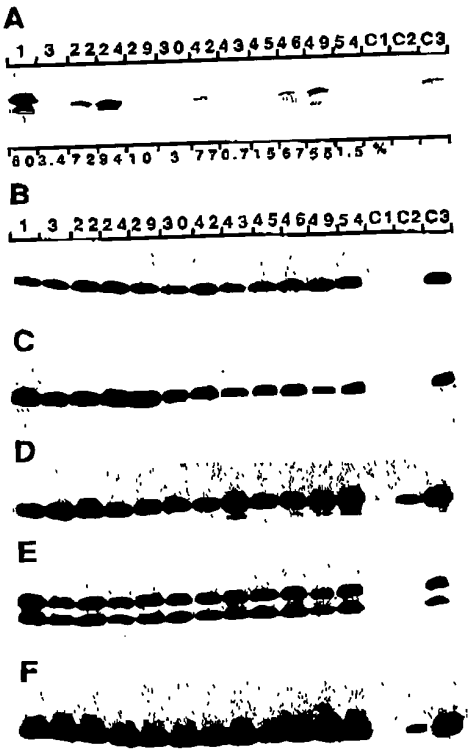

Fig. 3. Western-type analysis of total protein isolated from leaves of different transgenic and control potato plants. After isolation total protein (50 μg each except lanes C1, C2 and C3) was subjected to PAGE under denaturing conditions, transferred to nitrocellulose membranes and subsequently analysed for the presence of different proteins using antibodies against the following proteins: A: 10 kd protein of the water-splitting apparatus of photosystem II; B: 16 kd protein of the oxygen-evolving complex of photosystem II: C: 23 kd of the oxygen-evolving complex of photosystem II; D: 33 kd protein of the oxygen-evolving complex of photosystem II: E: 22 kd protein associated with photosystem II; F: D1 protein of the reaction centre of photosystem II. The numbering given above panels A and B identifies independent transgenic potato plants harbouring the construct encoding the anti-sense RNA to the 10 kd protein (cf. Figure 1). Lanes C1, C2 and C3 contain 0.5, 5 and 25 μg total protein isolated from leaves of a nontransformed control potato plant. The numbers given below the lanes in panel A indicate the relative amount of the 10 kd protein in percentage as compared with the level present in wild-type plants. In the case of the 10 kd and the 22 kd proteins, each two protein bands immunologically reacting with the respective antibodies are seen in the Western blot. For the 22 kd protein the higher molecular weight protein is a nonspecific protein band not related to the 22 kd protein. For the 10 kd protein, where a monospecific antibody raised against a fusion protein produced in *Escherichia coli* (Stockhaus, 1989) was used, the different bands recognized most likely represent proteolytic degradation products.

and older fully expanded leaves (cf. Materials and methods) thus excluding a possible accumulation of the 10 kd protein in older leaves (data not shown). The steady state protein levels of the 16 kd (Figure 3B), 23kd (Figure 3C) and 33 kd (Figure 3D) polypeptides which have been demonstrated to be components of the oxygen-evolving complex of photosystem II, are not affected by the suppression of the amount of the 10 kd polypeptide. The same result was obtained for a 22 kd protein which is associated with

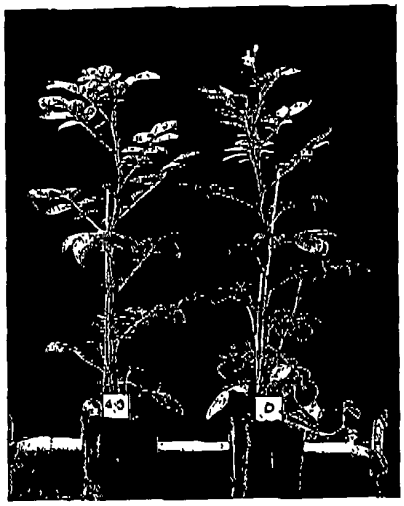

Fig. 4. Comparison of a wild-type (D) and transgenic potato plant (no. 43) when grown under normal white light conditions (150 W/m^2). Plant no. 43 expresses only 0.7% of the wild-type level of the 10 kd protein.

photosystem II (Figure 3E) and the D1 protein (Figure 3F) of the reaction centre of photosystem II.

Growth rates and morphological aspects

As mentioned above several independent transgenic plants containing severely reduced levels of the 10 kd protein show similar growth rates to the plants expressing wild-type levels of this protein.

In plant 43 (Figure 4) the amount of the 10 kd protein reaches only 0.7% of the wild-type level. Obviously however, no morphological difference between plant 43 and plants expressing wild-type levels of the 10 kd protein (Figure 4D) can be observed. This holds true for all transgenic plants analysed which contain reduced levels of the 10 kd protein (cf. Figure 5A).

In order to test whether under low light conditions plants containing a reduced level of the 10 kd protein would show a phenotype different from the wild-type, a set of plants was grown under limiting light conditions (15 W/m^2). Under these growth conditions the plants are characterized by elongated stems and small rudimentary leaves. Again no significant changes with respect to growth rate were observed between plants containing different amounts of the 10 kd protein (cf. Figure 5B).

Finally the ultrastructure of the chloroplasts of wild-type plants and plants showing a reduced level of the 10 kd protein was analysed. To this end plants were grown under two different light regimes, i.e. under normal white light (150 W/m^2) as well as under limiting light conditions (15 W/m^2). The rationale behind this experiment was that the depletion of the 10 kd protein could possibly lead to alterations of the chloroplastid ultrastructure such as the loss of the thylakoid stacking regions.

Figure 6 shows the electron micrographs of chloroplasts of a 10 kd protein depleted plant (Figure 6A: plant 43) and of a wild-type plant (Figure 6B) grown under normal white light (150 W/m^2) as well as of a 10 kd protein depleted plant (Figure 6C: plant 43) and a wild-type plant (Figure 6D) grown under limiting light (15 W/m^2).

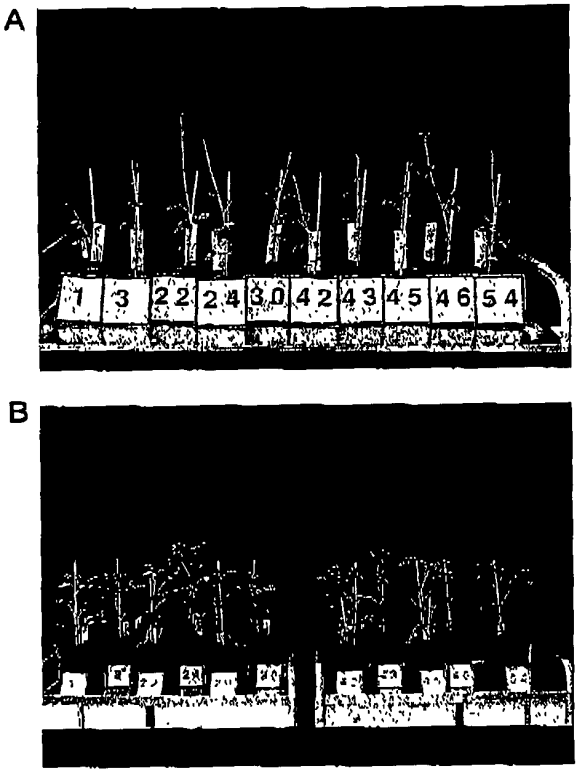

Fig. 5. Comparison of different transgenic potato plants varying widely in the residual level of the 10 kd protein (cf. Figure 3A) growing under low-light (A) and normal-light (B) conditions. Low light means 15 W/m^2, normal light means 150 W/m^2. Numbers identify independent transgenic potato plants harbouring the 10 kd anti-sense construct.

Chloroplasts of all plants grown under normal light conditions clearly display starch granules and grana stacks irrespective of the level of the 10 kd protein (Figure 6A and B). The ultrastructure of both plastid types of the plants is also very similar when grown under low-light conditions. In this case the plastids are characterized by the presence of a large number of grana regions (Figure 6C and D).

Functional analysis of the photosystem II

In order to analyse possible effects of the nuclear encoded 10 kd protein on the functional integrity of the PS II, two different kinds of experiments were performed: (i) the relaxation of the flash-induced enhancement in the fluorescence quantum yield, as determined in intact leaves; and (ii) the rates and characteristic oscillation pattern of O_2 evolution, as determined in an isolated thylakoid sample.

Figure 7 shows traces of transient fluorescence quantum yield changes induced by a train of two laser flashes in the dark-shaped leaves of control (dotted curve) and a 10 kd depleted plant (no. 3; full curve). The data reveal significantly slower relaxation kinetics in leaves of the transgenic plant lacking the 10 kd protein. This was observed in several independent experiments using leaves of different sizes and using independent vegetatively propagated plants of the same original transformant (data not shown). It is well established that the decay of the flash-induced fluorescence quantum yield reflects via a nonlinear relationship (Joliot and

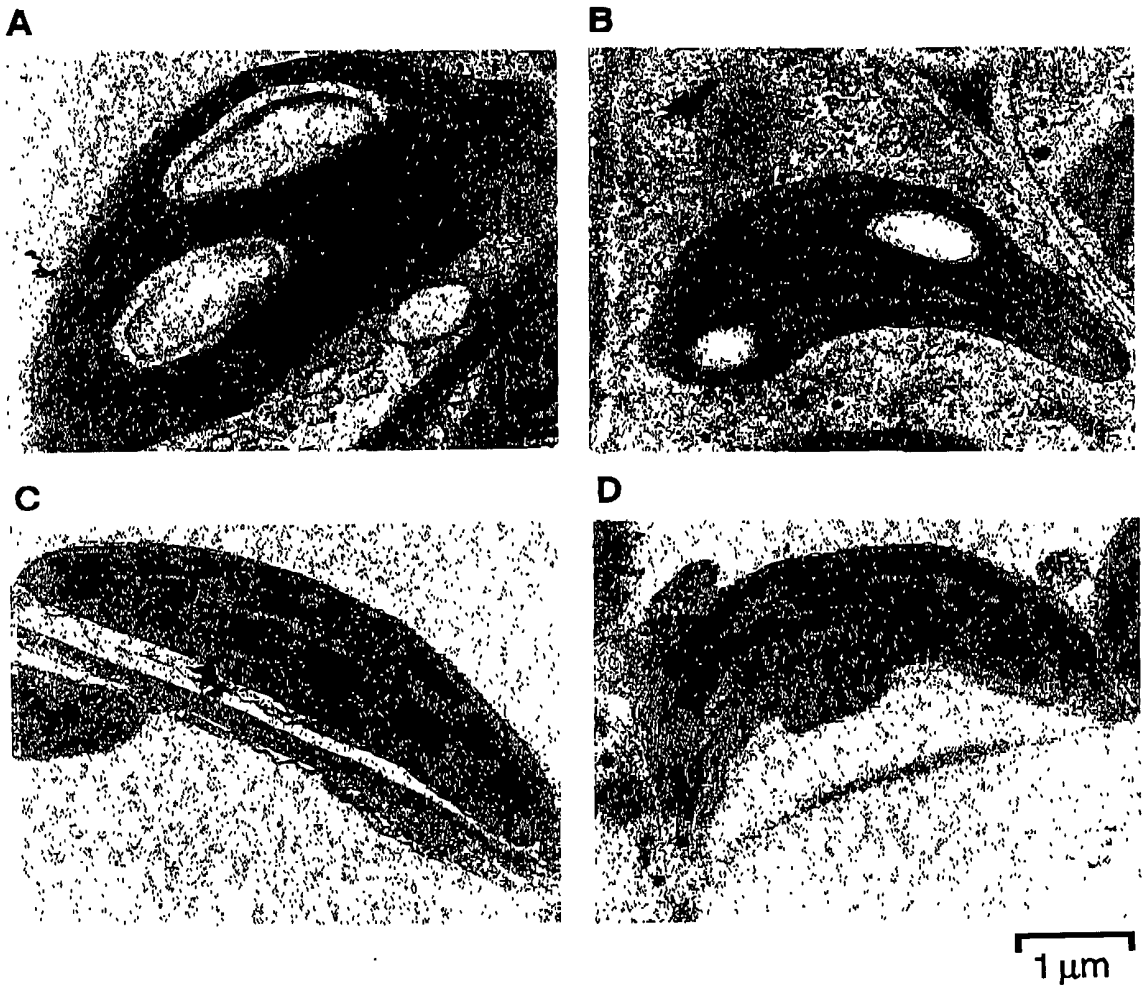

Fig. 6. Electron micrograph of chloroplasts of a wild-type (B and D) and transgenic potato plants containing only 0.7% of the wild-type level of the 10 kd protein (plant no. 43, panels A and C). Plants were kept either under low light (15 W/m^2; panels A and B) or normal white light (150 W/m^2; panels C and D) conditions.

Joliot, 1964) the reoxidation of Q_A- by Q_B and Q_B- (Robinson and Crofts, 1983). Therefore the results of Figure 7 clearly indicate that the elimination of the nuclear encoded 10 kd protein retards the the reoxidation of Q_A-.

With regard to the oxygen evolution measurements, the wild-type thylakoid samples exhibited maximum rates at 300–350 µmol O_2/mg Chl h in the presence of phenyl-*p*-benzoquinone (0.2 mM) and $K_3Fe(CN)_6$ (2mM) as the electron acceptor and 2mM NH_4Cl as uncoupler. Omission of the phenyl-*p*-benzoquinone led to much slower rates (~100 µmol O_2/mg Chl h).

In comparison with the wild-type under identical assay conditions the rates of O_2 evolution for 10 kd depleted transgenic plants (i.e. plants nos 3 and 43) were generally lower and showed a different pH dependence. At alkaline pH (>7.5), the initial rates were only 30–40% of those observed in the wild-type while at neutral and acid pH (down to 5.5) the differences were much less, the initial rates being 80% of the wild-type. The results are summarized in Table I.

As the steady state measurements are often complicated by acceptor limitations we also determined the O_2 yield induced by a train of short flashes in dark-adapted thylakoids in the absence of an exogenous electron acceptor. A typical period four oscillation pattern is observed which reflects the dioxygen formation via a sequence of four univalent redox steps at the catalytic site of water oxidation (for a recent review see Renger, 1987). As depicted in Figure 8, the overall oscillation pattern is not markedly disturbed in the thylakoid samples isolated from the 10 kd depleted transgenic plant. However, a close inspection of the data reveals that a higher damping occurs in this plant (i.e. transformant no. 3) compared with the wild-type. This phenomenon is very reproducible and was observed in different preparations of the same transformant as well as in preparations of a different transformant (plant no. 43; data not shown). Thus the lack of the 10 kd protein must also have some influence on the reaction pattern of the photosynthetic water cleavage.

Discussion

The photosynthetic apparatus of higher plants represents a very complex structure. It is composed of numerous different

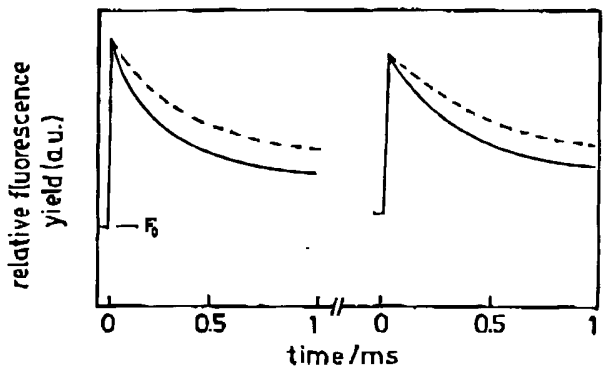

Fig. 7. Transient changes of fluorescence yield induced by a train of two laser flashes in intact leaves of wild-type (dotted curve) and 10 kd protein lacking transgenic potato plants. Dark time between the laser flashes: 1 s.

Table I. Altered pH dependence in the steady state rates of O_2 evolution of a 10 kd depleted transgenic potato plant (no. 3) in comparison with the wild-type.

pH	O_2 Evolution initial rate (−10 kd)/initial rate (+10 kd)
5.5	0.79
6.3	0.76
7.0	0.76
7.5	0.65
8.0	0.42

These data are based on three to five measurements from two different preparations of transformant no. 3. The error limit of the rates was ±10%. Similar rates were obtained for transformant no. 43 (data not shown).

proteins which are encoded by two different genomes, i.e. the plastome and the nuclear genome, which obviously asks for a tight interaction between the expression of both these genomes. Furthermore it has to react to a variety of environmental and developmental changes.

In order to understand the contribution and the role of the different polypeptides contained within this complex, one approach which has been used extensively in the past is the isolation of different structures depleted of one or more proteins and their *in vitro* analysis by biochemical and biophysical methods. This approach has its limitations and needs to be complemented by constructing *in vivo* complexes which are devoid of or at least heavily depleted by one and/or more polypeptides.

One way to overcome this problem is to screen for mutants. Though this approach has been successfully used especially in the case of the algae *Chlamydomonas*, it is not so easily feasible with higher plants especially when the mutant phenotype is unknown which is true for most mutations in any of the photosynthetic proteins. We therefore decided to use the anti-sense RNA approach to try to suppress the expression of a well-defined gene encoding the 10 kd protein of the water-splitting apparatus of photosystem II.

Expression of anti-sense RNA leads to efficient suppression of the 10 kd mRNA and protein amounts

In order to express the anti-sense RNA the full length cDNA was fused to the promoter of th 35S RNA of CaMV. This

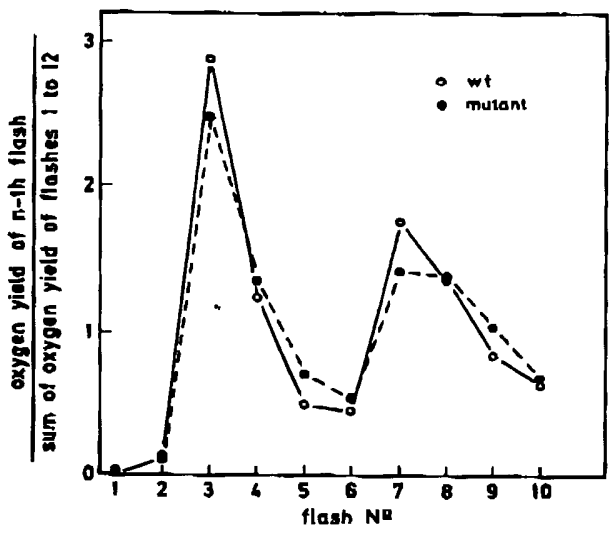

Fig. 8. Normalized oxygen yield as a function of flash number in dark-adapted thylakoids from wild-type (wt) and transgenic mutant potato plants. The data are normalized to the integral oxygen evolution due to flashes 1–12 of the train. Experimental details as described in Materials and methods.

promoter was chosen for two reasons. (i) As the 35S CaMV promoter is expressed in a variety of tissues and cells and is not restricted to photosynthetically active cells like the promoter of the 10 kd protein gene (Stockhaus *et al.*, 1989b), anti-sense RNA is already accumulating in the tissue before the promoter of the 10 kd gene is activated and sense RNA is produced. (ii) Due to the activity of the 35S promoter in root tissue, the level of anti-sense RNA can be determined in roots without possible interference due to the presence of sense RNA as in leaf tissue.

As described in Results, the expression of the 10 kd protein was suppressed to different levels in independent transgenic potato plants; in some transformants the steady state mRNA level reached only −1% of the wild-type level. Thus the expression of anti-sense RNA in transgenic potato plants reduces the expression of the 10 kd protein in a most effective manner. An important notion with respect to the mechanism by which anti-sense RNA induced inhibition works is the finding that there is no obvious correlation between the steady state level of the anti-sense RNA and the 10 kd gene steady state transcript levels.

The mechanism of the inhibitory effect of anti-sense RNA on the expression of the target gene is not understood. In principle anti-sense RNA could act at the level of transcription, processing and transport of the RNA out of the nucleus, at the level of translation or could generally affect RNA stability. Irrespective of the mechanism, if the anti-sense RNA interacts with the sense RNA, a bimolecular reaction has to be postulated. This should result in a concentration dependence of the observed inhibiting effect.

Concerning transgenic plants, anti-sense RNA expression has been used in few cases to inhibit the expression of an endogenous gene. Unfortunately the level of anti-sense RNA was not in all cases determined and compared with the sense RNA. A clear correlation between the inhibitory effect of the anti-sense RNA and its transcript levels has only been described by Delauney and colleagues (Delauney *et al.*,

1988) thus favouring the idea that the RNA is destabilized by a mechanism most likely involving heteroduplex formation. In the case of inhibition of the expression of the chalcone synthase gene by anti-sense RNA however, Van der Krol *et al.* (1988) described a rather complex pattern indicating a complex mechanism. Thus, different pigmentation patterns were observed in flowers of independent transgenic plants irrespective of the fact that these plants expressed the same steady state levels of chalcone synthase anti-sense RNA. On the other hand, cases were described where very low steady state levels of anti-sense RNA showed a pronounced effect on flower pigmentation (Van der Krol *et al.*, 1988). The data described in Results provide evidence for a more complex mechanism of anti-sense RNA inhibition. Although the fact that there is no clear correlation between the level of anti-sense RNA and suppression of the 10 kd gene expression does not exclude mechanisms involving heteroduplex formation between sense and anti-sense RNA, this assumption is not sufficient to explain these results. In order to explain our data, parameters have to be incorporated into a model. One possibility is that the integration site of the anti-sense construct with respect to the location of the target gene is of importance. If the inhibition of expression occurs very early after transcription of the sense RNA one might envision that different integration sites of the anti-sense RNA could lead to different local concentrations of the anti-sense RNA at the position of the target gene if the movement of the RNA within the nucleus is not diffusion-controlled, thus resulting in different efficiencies of suppression of the sense RNA.

Accumulation of chloroplastidic and nuclear encoded transcripts of genes involved in photosynthesis is unaffected by reduction of the 10 kd RNA and protein

Both photosystems I and II are composed of a large number of polypeptides encoded either in the nucleus or in the plastids thus requiring a tight coordination of gene expression. Recently, evidence for direct as well as indirect regulatory interactions between the nucleus and the plastids was obtained in different laboratories (reviewed in Taylor, 1989). Thus it has been shown that the state of chloroplast development drastically affects the expression of nuclear genes encoding chloroplastid proteins and it has been suggested that a plastid derived factor is involved in the transcriptional control of the expression of a number of genes encoding chloroplastidic polypeptides (Bradbeer *et al.*, 1979; Mayfield and Taylor, 1984; Oelmüller and Mohr, 1986; Batschauer *et al.*, 1986; Stockhaus *et al.*, 1989a). One possible mechanism would be a feedback-type regulation of certain transcripts and/or polypeptides involved in the photosynthetic process.

As shown in Figure 2 the steady state transcript levels of several plastid and nuclear genes analysed are not influenced significantly by the reduction of the 10 kd mRNA. This indicates that the transcription of these genes is not coupled to the expression of the 10 kd gene via a feedback control mechanism.

Effects of the 10 kd polypeptide depletion

In order to understand the role of the 10 kd protein, several experimental lines were followed. On the one hand plants showing a residual level of only 1% of the 10 kd protein were compared with wild-type plants with respect to the composition of other polypeptides belonging to photosystem II. This is especially important as one assumption based on reconstitution experiments is that the 10 kd protein might be necessary for the binding of the 23 kd polypeptide (Ljungberg *et al.*, 1986) which has been shown to play a regulatory role in oxygen evolution (Miyao and Murata, 1989). The analysis of the accumulation of some of the photosystem II polypeptides including the 23 kd shows that they are unaffected by the depletion of the 10 kd polypeptide. In a second line of experiments macroscopic parameters such as habitus, growth rates and assimilation capacity were determined for both wild-type plants and plants depleted in the 10 kd protein. As described in Results no obvious changes were observed. This indicates that the photosynthetic capacity of the transgenic plants depleted in the 10 kd protein is not significantly affected under the growth conditions used. Additional analysis performed at the microscopic level again revealed no change in the ultrastructural characteristics of the chloroplasts.

In a third type of experiment the functional integrity of the PSII complex of the wild-type and of plants depleted in the 10 kd protein was analysed by measurements of light-induced transient changes of the fluorescence quantum yield and of oxygen evolution. The fluorescence measurements clearly indicate that the lack of the 10 kd protein significantly retards the reoxidation of Q_A- by Q_B and Q_B-. As these functional redox groups are embedded in the heterodimer consisting of polypeptides D1 and D2 (for a review see Trebst, 1986) the results of this study provide evidence for an allosteric influence on the kinetics of acceptor side reactions as discussed previously (Renger *et al.*, 1981). The mechanistic implications of these findings will be analysed in more detail in a forthcoming paper.

The light saturated rates of oxygen evolution revealed a lower activity in thylakoids isolated from the transgenic plants lacking the 10 kd protein compared with those of the control. The extent of this diminishing effect greatly varied with pH. Therefore the 10 kd could act as a pH-dependent stabilizing polypeptide. A mechanically interesting phenomenon is the higher damping of the period four oscillation of oxygen yield in dark-adpated thylakoids from plants lacking the 10 kd protein.

An increase of the probability of misses could conceivably be due to a shift of the redox equilibrium comprising the couple Q_A/Q_A- and the plastoquinone bound to the Q_B site of polypeptide D1 in a way that leads to slightly modified properties of the Q_B site. This idea is supported by the fluorescence measurements described in this study but additional effects due to destabilizations of the donor site cannot be excluded.

Alternatively a general disorder introduced into the PSII complex by the lack of the 10 kd protein may slightly modify the properties throughout the PSII reaction sequence, without a particular target site of action. Detailed biophysical studies on the reduction kinetics of Q_A, the herbicide binding and the fluorescence induction curves are under progress to clarify this point.

In other organisms such as for example cyanobacteria or the green alga *Chlamydomonas reinhardtii*, photosystem II mutants are available for analysis of the assembly of photosystem II *in vivo* (Pakrasi *et al.*, 1989; Rochaix and Erickson, 1988; Kuchka *et al.*, 1989). Thus it has been

shown for *Chlamydomonas* that a deficiency of the nuclear gene product OEE1 (oxygene evolving enhancer) affects the stability of the photosytem II core, whereas the deficiency of the polypeptide OEE2 did not have any effect on the accumulation of the other photosystem II polypeptides Mayfield *et al.*, 1987a/b).

By using the anti-sense approach we have extended this kind of structural analysis of photosystem II to higher plants. This powerful approach will be very useful for the functional analysis of a number of different polypeptides of photosystem II which, like the 10 kd protein, are seemingly unique to photosystem II of higher plants.

Materials and methods

Material

DNA probes for the various nuclear and plastid genes were isolated from *Flaveria trinearvia* (M.Höfer and P.Westhoff, unpublished results). Antisera used are described in Oswald *et al.* (1990)

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (Maniatis *et al.*, 1982).

Transformation of potato plants and tissue techniques

The chimeric genes were inserted in the vector BIN19 (Bevan, 1984) and introduced into the *Agrobacterium tumefaciens* strain pGV2260 (Debluere *et al.*, 1985) by direct transformation according to Höfgen and Willmitzer (1988). The transformation and regeneration of *Solanum tuberosum* cv. Desiree plants was performed as described (Rocha-Sosa *et al.*, 1989).

Growth conditions and analysis of transgenic plants

Regenerated plants used for the biochemical, molecular and ultrastructural analyses were grown in a greenhouse under controlled temperature and light conditons (16 h light, temperature: 19°C; 8h dark, 10°C). Plants with a height of 60 cm were used for harvesting material. Young leaves means the fifth to seventh leaf (counting from top to bottom of the plant); old leaves means completely expanded, however, not yet senescent leaves of the plant.

Isolation of DNA and RNA from transgenic plants and their subsequent analysis by blot hybridizations were performed as described (Eckes *et al.*, 1986; Sanchez-Serrano *et al.*, 1987). The plant material was routinely harvested in the middle of the light period, i.e. at ~2 p.m.

Preparation of mesophyll protein fractions and Western blotting

Leaf tissue was homogenized in ice-cold buffer A (330 mM sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 2 mM EGTA, 5 mM dithiothreitol, 2 mM diethyldithiocarbamic acid, 50 mM Tris pH 7.5). The membrane fraction was collected by centrifugation and washed with buffer A. The membrane fraction was resuspended in buffer B (10% sucrose, 20 mM Na carbonate, 50 mM dithiothreitol, 1 mM PMSF, 2 mM diethyldithiocarbamate) and sonicated twice for 15s with a Branson sonicator. The protein concentrations of cell extracts were determined as described by Bradford (1976). Protein gel electrophoresis was performed according to Schägger and von Jagow (1987). Separated proteins were electrophoretically transferred to nitrocellulose membranes (PH79: Schleicher & Schüll) using a semi-dry blotting device (Pharmacia LKB, Freiburg). Immunodetection with antibodies, incubation with ^{125}I-labelled protein A (Amersham Buchler) and fluorography was performed as described by Westhoff *et al.*, (1985).

Fluorescence measurements

The flash-induced changes of fluorescence yield were measured as described in Renger *et al.*, (1988) using leaves as probing material.

Thylakoid isolation and O_2 measurements

Potato leaf material was homogenized in extraction buffer (330 mM sorbitol, 5 mM $MgCl_2$, 5 mM Na isoascorbate, 50 mM HEPES pH 7.5) with a Waring Blendor, passed through Miracloth and the chloroplasts were collected by centrifugation of 1 min at 1500 *g*. The chloroplasts were resuspended in lysis buffer (5 mM $MgCl_2$, 50 mM HEPES pH 7.5). The thylakoids were collected by centrifugation at 6000 *g* for 5 min and washed (330 mM sorbitol, 5 mM $MgCl_2$, 50 mM HEPES pH 7.5). The chlorophyll concentration was determined spectrophotometrically according to Arnon (1949). The rates of oxygen evolution were determined with a Clark-type electrode in a cuvette illuminated with white light of saturating intensity. The suspension contained: thylakoids (5 μg/ml Chl) 5 mM NaCl, 2mM NH_4Cl MES/NaOH and 2 mM $K_3Fe(CN)_6$ in the absence or presence of 1 mM phenyl-p-benzoquinone (Ph-p-BQ) as electron acceptor. The oxygen yield per flash induced by a train of saturating Xenon flashes (FWHM 10 μs) in dark-shaped samples was determined with a Joliot-type electrode as described in Hanssum *et al.*, (1985). These experiments were performed at pH 7.6 (50 mM Tricine/NaOH) in the presence of 5 mM $MgCl_2$ and 20 mM NaCl without electron acceptor.

Ultrastructure of the chloroplasts

Small leaf samples were embedded in Poly/Bed 812 Embedding Media (Polysciences, Inc.) as described by the supplier. Ultrathin sections were inspected by electron microscopy.

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Determination of Amylose: Amylop ctin Ratios of Starches

By J. G. Sargeant, High Wycombe

A method has been developed for the determination of amylose: amylopectin ratios of starches which offers an alternative to iodimetric methods. Starches solubilized in dimethylsulphoxide were debranched with isoamylase and the resulting linear components quantitated by gel permeation chromatography on a column of Sepharose CL-6B.

Bestimmung der Amylose: Amylopektin-Verhältnisse von Stärken. Es wurde eine Methode zur Bestimmung der Amylose: Amylopektin-Verhältnisse von Stärken entwickelt, die sich als Alternative zu iodometrischen Methoden anbietet. Die in Dimethylsulfoxid gelösten Stärken wurden mit Isoamylase entzweigt und die resultierenden linearen Komponenten durch Gelpermeationschromatographie auf einer Sepharose CL-6B-Säule quantitativ bestimmt.

1 Introduction

It is well established that starch consists of two chemically distinguishable polysaccharides, amylose and amylopectin. They are separable by a variety of techniques, the most common being the selective precipitation of amylose by complexing with *n*-butanol [1]. In the past, quantitative determinations of amylose by methods relying on its capacity for iodine absorption [2–4] have been used to estimate amylose: amylopectin ratios of starches. However, iodimetric methods can be unreliable due to the tendancy for amylose to retrograde in aqueous solution. Also, this type of determination is known to be affected by the presence of starch lipid [5]. It is evident that a more specific method is needed for the quantitative analysis of amylose and amylopectin.

Recently, enzymic methods have been developed to study the fine structure of amylopectin which use specific (1 → 6)-α-D-glucosidases which are collectively termed debranching enzymes. The two debranching enzymes most studied have been pullulanase [6] and isoamylase [7]. The latter has proved more useful as it completely debranches the amylopectin molecule to yield a population of linear chains that can be fractionated into two distinct molecular weight ranges [8]. This bimodal distribution of chains comprising A-chains (DP 15–20) and B-chains (DP ≃ 45) forms the basis for a revised model of the amylopectin molecule [9].

Starch granules can be completely solubilized in dimethylsulphoxide (DMSO) without any change in the primary structure of the polysaccharide [10]. Additionally, *Cytophaga* isoamylase is active in solutions containing up to 40% DMSO [11] and therefore the debranching of amylopectin is possible under conditions which minimise amylose retrogradation.

This paper describes a method for the determination of amylose: amylopectin ratios of three types of starch by DMSO solubilization of granules, isoamylase debranching and quantitative gel permeation chromatography of the linear fractions.

2 Materials and Methods

2.1 Materials

Lytic enzyme L1 from *Cytophaga* sp. was obtained from BDH Ltd. and β-amylase (analytical enzyme) from Wallerstein Co, Illinois. DEAE cellulose (DE52) was purchased from Whatman Biochemicals and Sepharose CL-6B from Pharmacia Fine Chemicals Ltd. DMSO (spectrophotometric grade) was obtained from Aldrich Chemical Co. All other chemicals were AnalaR grade chemicals, where possible, from BDH Ltd. or Sigma Chemical Co.

Waxy maize (*wx*) starch and amylomaize (*ae*) starch (Amylo 5, reputed to be 50% amylose) obtained originally from *M. S. Zuber*, University of Missouri, were kindly donated by *W. R. Morrison*, University of Strathclyde. Wheat starch was obtained from a commercial flour by aqueous washing from a dough.

2.2 Preparation of Debranching Enzyme

Isoamylase was partially purified from crude lytic enzyme preparation essentially by the method described by *Gunja-Smith* et al. [7] but with some modification:

Lytic enzyme (1 g) was gently dispersed in 10-mM Tris-HCl buffer, pH 8.0 (40 ml) at 4 °C and mixed for 15 min. The suspension was centrifuged (32,000 g) and the clear supernatant was passed through a column (1.6 × 10 cm) of DEAE cellulose (DE52) which had been previously equilibrated with the same buffer. The column eluate was monitored continuously with a UV analyzer (A_{280}) and the proteins not retained on the column were collected, dialyzed against distilled water (3 changes) and freeze-dried.

Freeze dried material (≃ 35 mg) from this isolation procedure, when assayed for isoamylase activity using a glycogen substrate [7], was usually found to possess an activity of 0.05–0.10 units/mg (one unit being defined as that amount of enzyme that releases 1 μ mole of glucose equivalent per minute at 37 °C).

2.3 Debranching of Starch with Isoamylase

To dry starch samples (15 mg) weighed into glass screw-capped vials was added 90% v/v aqueous DMSO (2 ml). The vials were carefully flushed with nitrogen, sealed and placed in an oven at 95 °C overnight (16 h) during which time the starch dispersed to give completely clear solutions. Sodium acetate buffer 40-mM pH 5.5 (6.9 ml) was then added and after mixing, 0.1 ml of isoamylase solution (5 mg/ml freeze dried preparation in acetate buffer) was added. The resulting solution was therefore 20% v/v DMSO, 30-mM Na-acetate buffer pH 5.5.

The debranching reaction was allowed to continue at 37 °C in a gyrotary water bath shaker until the reducing-end group value became constant (about 24 h). After this time 1 ml of 2.5-M KOH was added and the digestions were stored at 2 °C until required (not more than 2 days).

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2.4 Analysis of Debranched Starches

The linear starch fractions after debranching were fractionated by gel permeation chromatography on a column (1.6 × 70 cm) of Sepharose CL-6B. The column was eluted with 0.25-M KOH that had been previously degassed to remove dissolved oxygen (thereby minimising any possible alkaline degradation of the debranched starch components). For each chromatographic separation 1 mg (0.66 ml) of debranched starch was applied to the top of the column, and the column outlet was connected to the manifold of an autoanalytical system set up for total carbohydrate determination by the cysteine-sulphuric acid method [12]. The system comprised a Technicon multichannel pump and oil bath and a Vitatron photometer and recorder. Details of this autoanalytical system can be found in Figure 1. Cysteine-sulphuric acid reagent was prepared by dissolving L-cysteine hydrochloride (12.5 g) in 88% sulphuric acid (5 *l*). HCl gas was evolved during this stage.

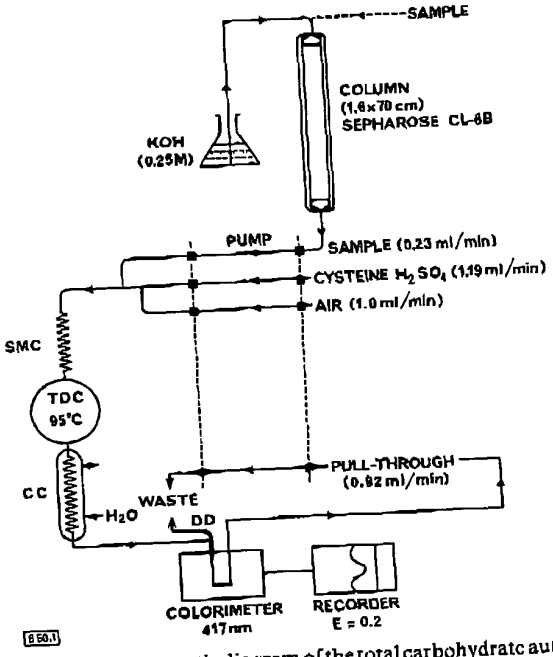

Figure 1. Schematic diagram of the total carbohydrate autoanalytical system. Pump tubes were flow measured Tygon tubes (Technicon). SMC-single mixing coil; TDC – 15 min time delay coil (10 m); CC – cooling coil, DD – debubbling device.

At a sampling flow rate of 0.23 ml/min each separation was complete in approximately 8.5 h after which the chart recorder peak areas were quantified by the cut and weigh method.

The linearity of response of the autoanalytical system over the range of detection used for analysis was confirmed with standard solutions of D-glucose within the range 0 – 30 µg/ml.

2.5 Degree of Polymerisation

The degree of polymeriz tion (DP) of the carbohydrates in column fractions was determined by the methods of *Sturgeon* [13] and *Richards* and *Whelan* [14].

2.6 Determination of Starch Lipid

Starch surface lipids were first removed by three successive washings (5 min each) with chloroform : methanol (2 : 1 v/v) at 25 °C. After drying (25 °C *in vacuo*) the true starch lipids were estimated as fatty acid methyl esters after acid hydrolysis and transesterification [15].

2.7 β-Amylolysis Limits

Determination of β-amylolysis limits of starches before and after debranching were carried out as follows:

Before debranching, 50 µl of starch dispersed in 90% v/v DMSO (375 µg) was added slowly to a stirred solution of 50-mM Na-acetate buffer pH 4.8 (9.95 ml), containing 5 units of β-amylase, (one unit being that amount of enzyme that will liberate 1.0 mg of maltose from starch in 3 min at pH 4.8. 20 °C). Liberated maltose was determined after complete β-amylolysis (5 h at 37 °C) by reducing-end group analysis using the neocuproine method [16] and total carbohydrate present in the digestions was determined by the cysteine-sulphuric acid method [12]. The extent of β-amylolysis was calculated by:

$$\% \text{ β-amylolysis} = \frac{\text{µg/ml reducing-end groups (as maltose)}}{\text{µg/ml total carbohydrate (as maltose)}} \times 100$$

The β-amylolysis limits of the debranched starches (in 0.25-M KOH) were determined after neutralization with HCl. This was done immediately before addition to the β-amylase digestions.

3 Results and Discussion

3.1 Debranching of Starches

Complete debranching of wheat, waxy maize and amylomaize starch was achieved with isoamylase under the experimental conditions used. This was confirmed by the high β-amylolysis limits obtained, as shown in Table 1, and indicated that the debranched starches were composed entirely of linear molecules that potentially could be fractionated on the basis of their molecular weights.

Table 1.
β-Amylolysis Limits of Starches Before and After Debranching.

Starch	β-Amylolysis limits (%)	
	before debranching	after debranching
Wheat	61.9	98.2
Waxy maize	57.3	98.8
Amylomaize	63.5	99.5

During debranching however, wheat and amylomaize starches produced a fine gelatinous precipitate which was shown to be a high molecular weight amylose fraction (*i. e.* eluted in the void volume). Linear amylose molecules are known to rapidly associate in solution to form molecular aggregates that exceed colloidal dimensions and precipitate [17]. This precipitated material however was found to be completely debranched since, after solubilization in 0.25-M KOH and subsequent neutralization, it gave high β-amylolysis limits (100.2% and 97.8% for wheat and amylomaize starch respectively). No precipitate was produced with debranched

waxy maize starch. The products of the debranching reactions were rendered totally soluble in 0.25-M KOH, this treatment facilitating the quantitative determinations of all the α-(1 → 4) linked linear molecular species present in the original starches as the linear and branched polysaccharides, amylose and amylopectin. Concentrations of KOH less than 0.25-M did not completely solubilize the reaction products and higher concentrations of DMSO (up to 40% v/v) for debranching, although slightly decreasing the quantity of insoluble material before the addition of alkali, gave little advantage as it reduced the activity of the debranching enzyme and caused precipitation problems upon subsequent addition of alkali.

3.2 Chromatography of Linear Fractions

Sepharose CL-6B was found to be an ideal medium for gel permeation chromatography of the range of linear components obtained from the debranched starches. Additionally this medium is stable in alkaline solutions up to pH 14 and therefore 0.25-M KOH could be used as the column eluant. Thus retrogradation of high molecular weight amylose fractions was prevented and quantitative recoveries of carbohydrates from the column could be obtained.
Alkaline degradation of carbohydrates during chromatography was not considered to be a problem but precautions were taken to exclude oxygen at all stages of analysis.
Figure 2 shows typical carbohydrate profiles obtained for the debranched starches after fractionation on a column of Sepharose CL-6B. For the purpose of amylose and amylopectin quantitation in wheat and amylomaize starches, the recorder traces were cut at a point (denoted by arrow) which divided amylose and amylopectin. This point was estimated from the waxy maize starch profiles, as the beginning of amylopectin elution was well defined owing to the absence of amylose in this starch. The results of six replicate analyses for each starch are given in Table 2.
Fraction I (Fig. 2, WS, WM, AM) represents the amylose component of the starches (DP > 135) and for wheat and amylomaize starch shows a wide range of chain lengths, the majority being eluted in the void volume (*i. e.* DP > 550). Amylomaize had a larger proportion of linear chains below DP 550 than wheat. Possibly these fairly short amylose molecules were derived from the debranching of partially branched amylose species as it has been suggested that amylose exists as a heterogeneous material comprising a slightly branched intermediate fraction [18]. They are unlikely to be a result of hydrolysis (either amylolytic or alkaline) of longer chain amylose as elution profiles were unchanged when either different quantities of enzyme preparation were used for debranching or higher concentrations of KOH used for elution.
Waxy maize starch possessed only a very small quantity of totally excluded carbohydrate (0.9%). This confirmed that isoamylase has the ability to completely debranch starches composed almost entirely of amylopectin.
Fractions II and III (Fig. 2) represent the amylopectin components of the starches after debranching (DP < 135). The profiles show a bimodal distribution of chain length which corresponds to the A-chains and B-chains described previously [9]. For wheat and waxy maize starch the average DP of fraction III (A-chains) and fraction II (B-chains) was found to be 18 and 60 respectively. In addition the A : B chain ratio was approximately the same for both starches. However, the average DP of fractions III and II in amylomaize was found to be 15 and 50 respectively and the A : B chain ratio was approximately 1 : 1. This suggests distinct structural differences in the amylopectin component of the starch.

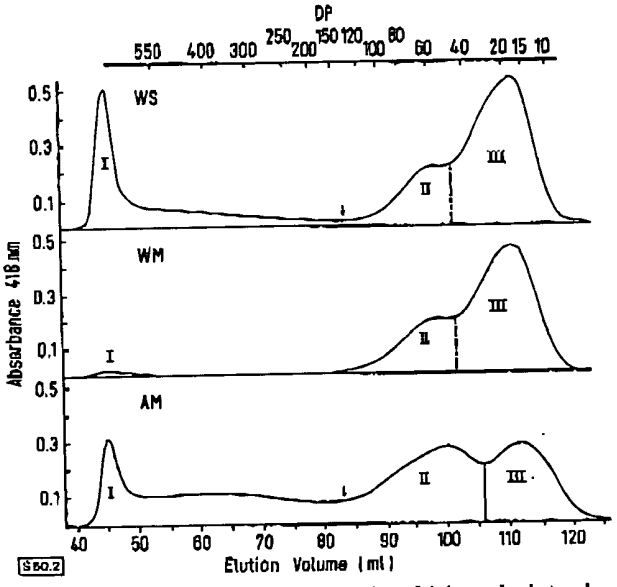

Figure 2. Typical carbohydrate profiles of debranched starches by gel permeation chromatography on a column (1.6 × 70 cm) of Sepharose CL-6B. WS = wheat starch; WM = waxy maize; AM = amylomaize; ↓ denotes point where trace was divided for the calculation of ratio.

Table 2.
Amylose : Amylopectin Ratios and Corresponding Lipid Levels of the Three Starches. The Results for Amylose and Amylopectin Represent the Mean of Six Replicate Determinations (Limits of Error Calculated for 95% Confidence Limits).

Starch	Amylose : Amylopectin Ratio (%)	Lipid (g FA/kg starch)
Wheat	28.7 : 71.3 ± 1.08	4.21
Waxy maize	0.9 : 99.1 ± 0.14	0.44
Amylomaize	37.8 : 62.2 ± 1.36	6.45

3.3 Starch Lipid Levels

Cereal starches can contain up to 1% lipid most of which is thought to be present inside the starch granule as an amylose inclusion complex [19]. In wheat the starch lipids are mainly lysophospholipids whereas in maize the free fatty acids predominate [20]. It has been suggested that the starch lipid regulates the amylose : amylopectin ratio of starch by preventing branching enzyme acting on the amylose-lipid complexes during synthesis of the granule [21].
The results in Table 2 tend to support this view since the quantity of starch lipid was found to be directly proportional to the amylose content of the starches.

Summary

A method has been developed for the determination of the amylose and amylopectin content of starches. Wheat, waxy maize and amylomaize starches were solubilized in DMSO and completely debranched using a bacterial isoamylase that was isolated from a crude *Cytophaga* preparation by DEAE cellulose chromatography. The resulting linear starch components were quantitated by gel permeation chromatography

on a Sepharose CL-6B column by eluting with KOH solution. Column eluates were monitored continuously using a total carbohydrate autoanalytical system and amylose:amylopectin ratios were calculated from the carbohydrate profiles by measurements of peak areas.
Wheat starch (28.7% amylose) and waxy maize starch (0.9% amylose) contained amylopectin components with similar A-chain and B-chain sizes and ratios whereas amylomaize showed a different amylopectin composition and contained amylose that, after debranching, had a significantly higher proportion of low molecular weight species than did wheat starch.
Starch lipid levels were found to be related to the amylose content of the starches.

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Infrared and X-Ray Analysis of Hydroxyethyl Starch

By S. I. El-Hinnawy, H. M. El-Saied, A. Fahmy, A. E. El-Shirbeeny and K. M. El-Sahy

The effect ob substituted groups in hydroxyethyl starches with variable degrees of substitution on the conformation of starch molecules was investigated by the infrared and X-ray analysis. The highest substitution with alkoxyl groups caused the shifting of the equatorial anomeric groups of glucose units to the axial position due to strain effect in the rings of monomers. The X-ray analysis showed that the volume of unit cell of starch was increased 4.3% with the highest substitution of corn starch. This improved the penetration of dye molecules in the coating of textiles.

Infrarot- und Röntgenanalyse von Hydroxyethylstärke. Die Wirkung substituierter Gruppen in Hydroxyethylstärken mit unterschiedlichem Substitutionsgrad auf die Konformation der Stärkemoleküle wurde durch Infrarot- und Röntgenanalyse untersucht. Die höchste Substitution mit Alkoxylgruppen verursachte den Übergang der äquatorialen anomeren Gruppen der Glucoseeinheiten in die axiale Stellung aufgrund der Spannung in den Monomerringen. Die Röntgenanalyse zeigte, daß das Volumen der Stärke-Einheitszelle bei der höchsten Substitution von Maisstärke um 4,3% vergrößert war. Dadurch wurde das Eindringen von Farbstoffmolekülen in Textilbeschichtungen verbessert.

1 Intr ducti n

It is well known that the most widely utilized starch ether is hydroxyethyl starch which is prepared by the reaction of alkaline starch and ethylene oxide [1, 2]. Hydroxyethyl starch of low degree of substitution is used in paper and textile industries [3, 4, 5], as a blood volume expander and as a cryophylatic agent for erythrocytes [6, 7]. This leads to the

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Amylose Content and Chain Profile of Amylopectin from Normal, High Amylose and Waxy Barleys

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The amylose content and the chain profile of amylopectin from normal, waxy and high amylose barley starches were determined after enzymatic debranching and gel permeation chromatography and the degree of branching of the amylopectin was analysed by ^{1}H-n.m.r. spectroscopy. The normal barley starch contained around 30%, the high amylose around 40% and the waxy starch 9% amylose. The amylopectin of the high amylose starches had longer chains than those of the normal or waxy starches, especially in the molecular weight interval 5,400–8,000, but less of those below 2,400 in molecular weight. The chain length of amylopectin from high amylose barley was on average 5 units longer than those of normal or waxy barleys.

Amylosegehalt und Kettenprofil des Amylopektins aus normalen, amylosereichen und wachsigen Gersten. Der Amylosegehalt und das Kettenprofil des Amylopektins aus normalen, wachsigen und amylosereichen Gerstenstärken wurde bestimmt nach enzymatischer Entzweigung und Gel-Permeationschromatography, und der Verzweigungsgrad des Amylopektins wurde durch ^{1}H-NMR-Spektroskopie analysiert. Die normale Gerstenstärke enthielt um 30%, die amylosereiche um 40% und die wachsige Stärke um 9% Amylose. Das Amylopektin der amylosereichen Stärken hatte längere Ketten als das der normalen und wachsigen Stärken, besonders im Molekulargewichtsbereich von 5.400–8.000, jedoch weniger lange als diejenigen unterhalb 2.400 im Molekulargewicht. Die Kettenlänge des Amylopektins aus amylosereicher Gerste war durchschnittlich um 5 Einheiten länger als die aus normalen oder wachsigen Gersten.

1 Introduction

Barley is one of the most important cereal crops of the world. In Sweden barley is today mainly used as a feed grain but also finds substantial application in the brewing and food industry. A broad range of amylose and amylopectin contents in cereal starch is known only in mutant lines of the diploid species, barley, maize, sorghum and rice [1, 2]. The amylose content of high amylose barley is usually about 40% and of the waxy barley types below 10% [1, 3–6]. The ratio of amylose/amylopectin is important for both the nutritional and technological properties of the starch. The *in vitro* amylolysis rate of starch following autoclaving or boiling of the material, was lower in a high amylose barley than in a normal or waxy barley [7, 8]. The formation of enzymes resistant starch (RS) was positively correlated to the amylose/amylopectin ratio in this material. Analyses have shown that the main part of RS consists of retrograded amylose. This type of RS is formed in food products which are processed at a high moisture level, like cooking, baking and autoclaving [9, 10].

Amylose is necessary for the formation of bread crumb structure [11, 12]. Retrogradation of amylopectin is mainly responsible for bread staling [11]. The retrogradation rate of amylopectin has been shown to be dependent of increased chain lengths [13–15]. The high amylose types of maize and rice have a larger proportion of the longer chains of amylopectin but also longer average chain lengths than their corresponding normal or waxy types [16–20]. However, *Tester* et al. [5] found no differences in chain lengths of amylopectin from normal, waxy and high amylose barley starches when analysed by gel permeation chromatography (GPC) on Sepharose CL-6B after enzymatic debranching. Similar analysis of starches from normal and waxy barley types also did not show any major differences in amylopectin chain lengths [6, 21].

In the present investigation the amylose content and chain length distribution of the amylopectin of normal, waxy and high amylose barleys were determined by GPC. The chain length distribution was confirmed by analysis of the degree of branching by ^{1}H-n.m.r. A preliminary account of a part of this work was recently presented [22].

2 Materials and Methods

2.1 Materials

Five barley types were used; Golf, a Swedish covered variety with a normal ratio of amylose/amylopectin, waxy Compana, a covered variety with low amylose content, and Glacier, a covered variety with high amylose content, were obtained from the Swedish breeding company, Svalöf Weibull AB. Normal Glacier a covered variety with normal amylose content and naked high amylose Glacier were kindly donated by Dr. *C. W. Newman*, Montana State University, USA. The barley samples were ground in a Cyclotec 1093 Sample Mill (Tecator AB, Höganäs, Sweden) to pass a 0.5mm screen. The ground material was steeped in ethanol, homogenized and filtered. The procedure was repeated several times until no starch granules could be observed by microscopic examination of the filtrate. The crude starch preparation was purified according to *Karlsson* et al. [23].

2.2 Methods

Debranching with isoamylase and gel permeation chromatography in 0.25M potassium hydroxide of the debranched starch on a Fractogel TSK HW-50 (S) (Merck), a Superose 6 (Pharmacia) or on a Sepharose CL-6B (Pharmacia) column (1.5×91cm, flow rate 9ml/h, fraction size 1.1ml) was performed as described by *Torneport* et al. [24]. The void volume and the total volume of the chromatographic systems were determined by the amylose and glucose peak, respectively. Dextran standards T40, T10 and dextrans with $\bar{M}w$ 5,400 and 1,040Daltons (Pharmacia) were used as calibration substances and apparent molecular weights of degraded amylopectin were calculated with these standards. The degree of branching of the starch was determined by ^{1}H-n.m.r. according to *Salo-*

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monsson et al. [25]. All analyses were performed in at least duplicate.

3 Results

3.1 Amylose content and proportion of unit chains of the amylopectin

In this study the barley starches were debranched with isoamylase and then analysed by GPC using a column of either Fractogel TSK HW-50, Sepharose CL-6B or Superose 6. The elution profiles from the analyses by Fractogel TSK HW-50 are given in Figure 1 and the calculated results are presented in Table 1. Fraction I which was eluted first comprised the long linear chains from amylose and fractions II and III were comprised of the longer and shorter chains, respectively, from amylopectin. The starch in normal barleys contained 28 and 30% amylose and both the covered and naked high amylose barley starches contained around 40% amylose whereas the waxy type used had 9% amylose. The ratio of fractions III/II varied between 3.2 to 3.7 for the five barleys. The chromatographic

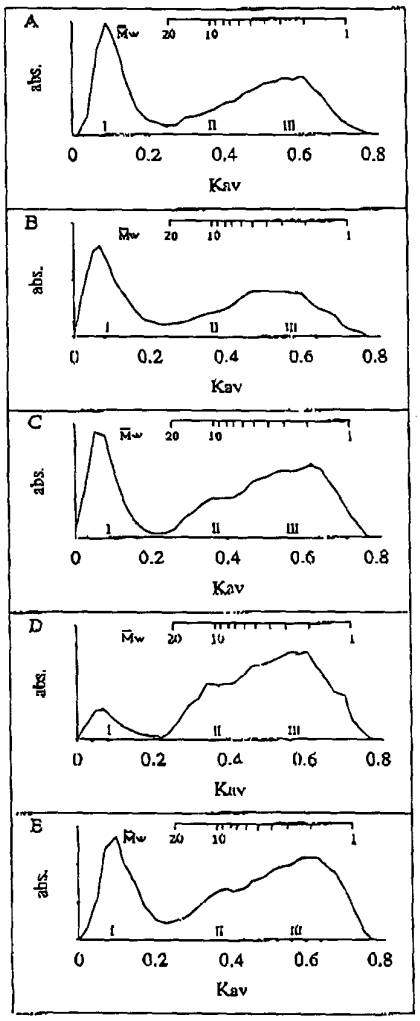

Fig. 1. Gel permeation chromatography elution profiles of debranched starches on a Fractogel TSK HW-50 column. A) Glacier high amylose naked, B) Glacier high amylose covered, C) Glacier normal, D) waxy Compana and E) Golf normal.

Table 1. Fractions (weight %) from Chromatography Elution Profiles (Fractogel TSK HW-50) of Debranched Starches from Five Genotypes of Barley.

Barley genotype	Fractions from debranched starch			
	I[a]	II[b]	III[c]	ratio III/II
Glacier high amylose naked	39	13	48	3.7
Glacier high amylose covered	40	13	47	3.6
Glacier normal	30	15	55	3.7
Waxy Compana	9	22	69	3.2
Golf	28	16	56	3.5

[a] First eluting component, amylose.
[b] Second fraction, longer chains from amylopectin.
[c] Last fraction, shorter chains from amylopectin.

analyses by Sepharose CL-6B and by Superose 6 gave nearly identical values as above for the amylose content and the proportions of the long and short unit chains of amylopectin for the analysed samples. Examples of the elution profiles from Sepharose CL-6B are given in Figure 2.

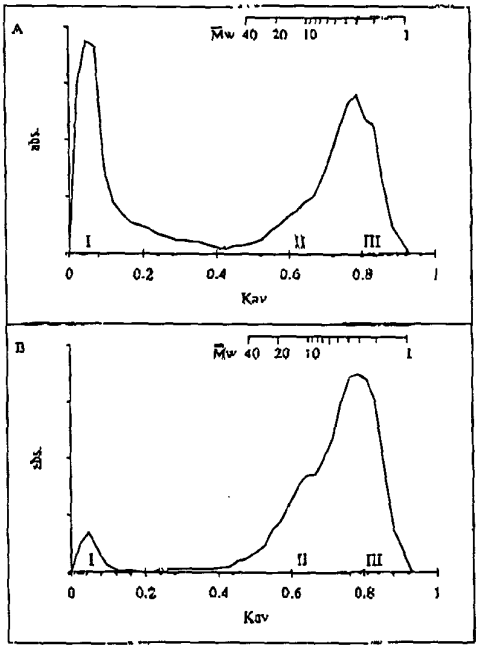

Fig. 2. Gel permeation chromatography elution profiles of debranched starches on a Sepharose CL-6B column. A) Glacier high amylose covered and B) waxy Compana.

3.2 Molecular weight of the unit chains of amylopectin

The distribution of the unit chains of amylopectin according to molecular weight obtained from the calibrated Fractogel TSK HW-50 column is given in Table 2. About one third of the debranched amylopectin material of the waxy and both the normal types was below 2,400 Daltons in molecular weight. However, in both the high amylose barleys, only one fifth of the material was found in this low molecular weight area. Instead, a larger proportion of the amylopectin unit chains from the latter barleys were found in the area between 5,400 and 8,000 Daltons in molecular weight. This means that the unit chains are longer in amylopectin from the high amylose bar-

Table 2. Relative Distribution (weight %) of Different Chain Lengths Analysed on a Fractogel TSK HW-50 Column and Degree of Branching (molar basis) in Amylopectin from Five Genotypes of Barley.

Barley genotype	14×10^3 <mol wt <Fr I[a]	8×10^3 <mol wt <14×10^3	5.4×10^3 <mol wt <8×10^3	3×10^3 <mol wt <5.4×10^3	2.4×10^3 <mol wt <3×10^3	mol wt <2.4×10^3	Branch points[b]
Glacier high amylose naked	4.1	10.0	23.3	24.6	17.6	20.4	4.3
Glacier high amylose covered	5.7	12.2	20.9	20.5	20.1	20.6	4.2
Glacier normal	5.4	11.7	14.5	20.1	14.5	33.8	5.3
Waxy Compana	7.2	13.2	14.6	18.7	14.0	32.3	5.4
Golf	7.0	11.8	12.3	16.4	13.1	39.4	5.4

a) First eluting component, amylose.
b) Determined by n.m.r.

leys compared to the normal or the waxy types investigated here.

3.3 Degree of branching

^{1}H-n.m.r. was used to determine the degree of branching of the isolated starches and the results are given in Table 2. The degree of branching of the amylopectin from the normal and waxy types were 5.3 to 5.4 whereas the two high amylose types had only 4.2 or 4.3 molar percentage of the anhydroglucose units involved in branches.

4 Discussion

The amylose content and the chain profile of the amylopectin of five different barley starches were examined by GPC after debranching with isoamylase. For dextran Fractogel TSK HW-50 (S) has a fractionation range of 500–20,000 Daltons, according to the supplier, whereas Sepharose CL-6B has a fractionation range of 10,000–1,000,000 Daltons. Superose 6 is a cross-linked agarose-based gel optimized for high performance GPC of biomolecules with a fractionation range similar to that of Sepharose CL-6B. The separation between the amylopectin chains and the amylose was better on the Sepharose CL-6B column than on the Fractogel column (cf Fig. 1 and Fig. 2). The elution pattern on Superose 6 was similar to that from the Sepharose gel, but the analysis was much quicker taking less than 2h per sample. Compared to the other two gel types Fractogel TSK HW-50 has a better resolution in the molecular weight range of the amylopectin chains.

The amylose content of the barley starches calculated from the GPC analyses was 9–12% higher in the high amylose than in the normal types. This is in accordance with results from earlier studies [3, 5, 7, 8]. The amylose content in the waxy barley used was 9% which is a value in the range reported for different waxy barley types [3].

The chain profiles of the amylopectins examined by different GPC systems revealed two main components, the longer and the shorter amylopectin chains. A trimodal distribution of the amylopectin chains was indicated for some of the samples and this is in agreement with previous studies on barley starch [5, 15, 21]. All distributions showed overlapping peaks and the peak areas were measured following to the previous convention [18]. The ratios of fraction III/II obtained in this study (Table 1) are very well within the range of the values of 3.3–4.4 obtained in previous investigations of normal or waxy barleys [6, 21]. *Tester* et al. [5] who used a Sepharose CL-6B column obtained somewhat lower values however and also found a slightly higher ratio for the high amylose Glacier amylopectin compared to the normal or waxy types used.

High amylose types of maize and rice have a low ratio, 0.8–1.6, of short/long amylopectin chains, and the chains are about 10 anhydroglucose units longer than normal [16–19]. In the present investigation made on the Fractogel column, the proportion of chains in the molecular weight range 5,400–8,000 Daltons was higher while in the range below 2,400 Daltons in molecular weight this proportion was lower, for the two high amylose barleys compared to the normal or waxy types (Table 2). In the analyses by the Sepharose CL-6B or Superose 6, which do not have as good separation in the low molecular weight area as the Fractogel, the increase in chain lengths could not be established. *Banks* and *Muir* [1] observed an average chain length of about 21 and 25 for isolated amylopectin from normal and high amylose barley, respectively.

However, the difference in that study was attributed to contaminant material in the amylopectin preparation. The degree of branching of the starches was obtained from the ^{1}H-n.m.r. spectra. If it is assumed that the amylose fraction did not contribute with any branch points, the figures in Table 2 could be directly calculated from the n.m.r. data. Amylopectin from the normal or waxy barley starches had as an average a branch point on every 18.5–18.9 anhydroglucose unit whereas every 23.3–23.8 unit was branched in the amylopectin from the high amylose barley starches. This means that the chains of the amylopectin from the high amylose barley starches are about 5 anhydroglucose units longer than in the normal or waxy barleys. These results are in agreement with the molecular weight distributions analysed by the Fractogel column. Thus the increase in chain length of the amylopectin of high amylose barley was not as pronounced as in high amylose rice or maize (about 10 units).

In high amylose rice or maize atypical starch materials have been detected. These are seen as an intermediate fraction in analyses of debranched total starch and/or an amylopectin with extra long chains (CL 85–180) from isolated amylopectin [16, 19, 20]. In the present investigation no differences in the amount of intermediate fraction (between fraction I and II in the GPC analyses) were found between the barley types. The amylopectin from the barley starches was not isolated in the present investigation so the absence of extra long chains, which elute from the GPC column with the amylose, could not be excluded. However, the degree of branching determined by n.m.r. would probably decrease even further if considerable amounts of very long chains were present in the high amylose barley starches.

Barley has a high potential for different food usages and the chain length distribution of amylopectin is important for the properties of food. High amylose barley with longer amylopectin chains could probably be used when a firmer texture and resistance to disintegration of the product is desired. In rice [26] these properties were correlated to longer exterior chains of the amylopectin. Reduced swelling or solubility of the starch is observed in high amylose cereal types [3, 5, 26]. In a recent study [8] it was discussed if this was one of the reasons

for increased satiety after meals when high amylose barley was used instead of normal or waxy barley.

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Colorimetric Method for Determination of Sugars and Related Substances

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Simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl ethers with free or potentially free reducing groups, give an orange-yellow color when treated with phenol and concentrated sulfuric acid. The reaction is sensitive and the color is stable. By use of this phenol-sulfuric acid reaction, a method has been developed to determine submicro amounts of sugars and related substances. In conjunction with paper partition chromatography the method is useful for the determination of the composition of polysaccharides and their methyl derivatives.

COLORIMETRIC tests for reducing sugars and polysaccharides have been known for a considerable time. The reagents such as 1-naphthol (*33*) for carbohydrates in general; benzidine for pentoses and uronic acids (*27, 49, 50*); naphthoresorcinol for uronic acids (*61*); and resorcinol (*43*), naphthoresorcinol (*39*), and resorcinol disulfonic acid (*31*) for ketoses are well-known examples of colorimetric tests that may be carried out in acid solution. Such tests as these and modifications of them using aromatic amines and phenols (*4, 22, 38*) have recently gained added importance since the extensive development of partition chromatography for the separation and characterization of minute amounts of sugars and their derivatives (*1, 4, 8, 11, 12, 17, 18, 21–23, 26, 36, 39, 47*). Polyols and carbohydrates with a reducing group may be detected by the Tollens silver reagent (*39, 52*), perhaps one of the best reagents in the art of chromatography. Reducing sugars are also detectable by picric acid (*7, 17*), 3,4-dinitrobenzoic acid (*5*), 3,5-dinitrosalicylic acid (*6, 32, 48*), *o*-dinitrobenzene (*17, 40*), and methylene blue (*54*), while diazouracil is said to be specific for sucrose as well as oligosaccharides and polysaccharides containing the sucrose residue (*42*).

Volumetric procedures involving the use of potassium ferricyanide (*19*), ceric sulfate (*45*), copper sulfate (*16, 44*), and sodium hypoiodite (*20*) are applicable to the determination of small amounts of reducing sugars after separation by partition chromatography. However, experience shows that these methods require considerable skill and are time-consuming and sensitive to slight variation in the conditions.

The anthrone (*13, 14, 28, 34, 35, 58*) and the 1-naphtholsulfonate (*10*) reagents are excellent for standard sugar solutions (*34*), but, when applied to the analysis of sugars separated by partition chromatography, the presence of only traces of residual solvent developer may render them useless. Most sugars can be separated on filter paper by a phenol-water solvent (*39*), but they cannot then be determined by the anthrone reagent because residual phenol, held tenaciously in the paper, interferes with the green color produced by the anthrone reagent. Moreover, the anthrone reagent is expensive and solutions of it in sulfuric acid are not stable (*30, 34*). The anthrone method also suffers from the disadvantage that, while it is satisfactory for free sugars and their glycosides, it is of limited use for methylated sugars and the pentoses. Although butanol–propionic acid–water is an excellent solvent for separating the disaccharides (*4*), the residual propionic acid interferes with the 1-naphtholsulfonate method. Aniline phthalate (*38*) and aniline trichloroacetate (*17*) have been utilized for the colorimetric determination of sugars and their derivatives (*2, 3*); these reagents, however, are unsatisfactory for ketoses.

Phenol in the presence of sulfuric acid can be used for the quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharides, and polysaccharides (*15*). This method is particularly useful for the determination of small quantities of sugars separated by paper partition chromatography with the phenol-water solvent and also for those sugars separated with solvents which are volatile—e.g., butanol-ethanol-water (*39*), ethyl acetate–acetic acid–water (*26*), or methyl ethyl ketone–water (*4, 39*). The method is simple, rapid, and sensitive, and gives reproducible results. The reagent is inexpensive and stable, and a given solution requires only one standard curve for each sugar. The color produced is permanent and it is unnecessary to pay special attention to the control of the conditions.

DETERMINATION OF CONCENTRATION OF PURE SUGAR SOLUTIONS

Reagents and Apparatus. Sulfuric acid, reagent grade 95.5%, conforming to ACS specifications, specific gravity 1.84.

Phenol, 80% by weight, prepared by adding 20 grams of glass-distilled water to 80 grams of redistilled reagent grade phenol. This mixture forms a water-white liquid that is readily pipetted. Certain preparations have been known to remain water-white after a years' storage, while others turn a pale yellow in 3 or 4 months. The pale yellow color that sometimes develops does not interfere in the determination, inasmuch as a blank is included.

Coleman Junior, Evelyn, Klett-Summerson, or Beckman Model DU spectrophotometers. All were used with satisfactory results in this investigation.

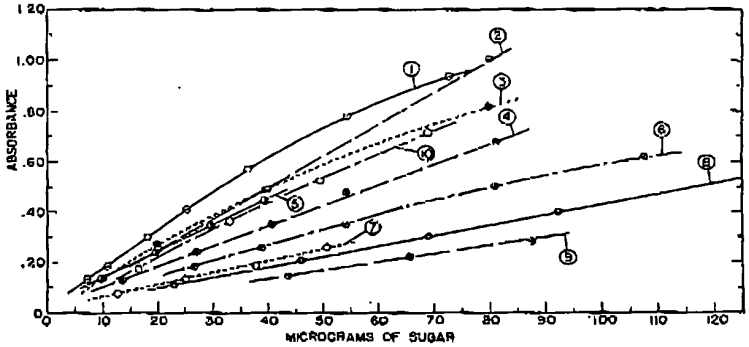

Figure 1. Standard curves

1. D-Xylose, Coleman Jr., 480 mμ, 17 mg. of phenol
2. D-Mannose, Beckman Model DU, 490 mμ, 40 mg. of phenol
3. D-Mannose, Evelyn, filter No. 490, 40 mg. of phenol
4. D-Galactose, Coleman Jr., 490 mμ, 33 mg. of phenol
5. L-Arabinose, Coleman Jr., 480 mμ, 17 mg. of phenol
6. D-Galacturonic acid, Coleman Jr., 485 mμ, 17 mg. of phenol
7. L-Fucose, Coleman Jr., 490 mμ, 40 mg. of phenol
8. D-Glucurone, Coleman Jr., 485 mμ, 17 mg. of phenol
9. 2,3,4,6-Tetra-*o*-methyl-D-glucose, Coleman Jr., 485 mμ, 17 mg. of phenol
10. D-Glucose, Beckman Model DU, 490 mμ, 100 mg. of phenol

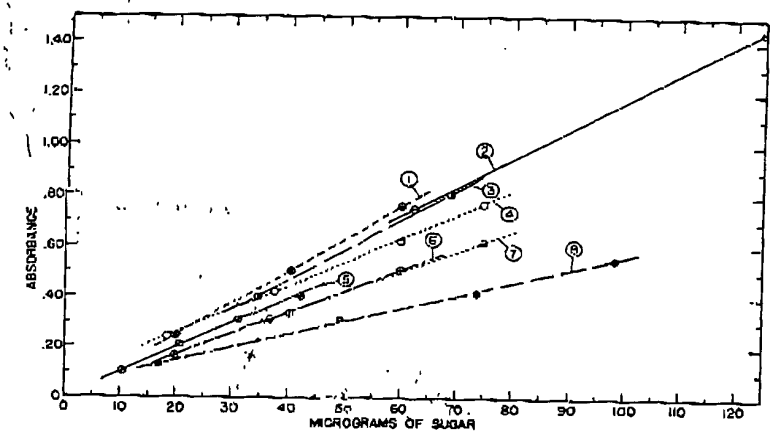

Figure 2. Standard curves

1. Sucrose, Beckman Model DU, 490 mμ, 100 mg. of phenol
2. Potato starch, Beckman Model DU, 490 mμ, 100 mg. of phenol
3. Dextran from *Leuconostoc mesenteroides* strain NRRL 512, Beckman Model DU, 490 mμ, 108 mg. of phenol
4. D-Glucose, Evelyn, filter No. 490, 80 mg. of phenol
5. L-Rhamnose, Coleman Jr., 480 mμ, 40 mg. of phenol
6. Raffinose, Beckman Model DU, 490 mμ, 100 mg. of phenol
7. D-Fructose, Beckman Model DU, 490 mμ, 200 mg. of phenol
8. 2-Deoxy-D-ribose, Coleman Jr., 480 mμ, 80 mg. of phenol

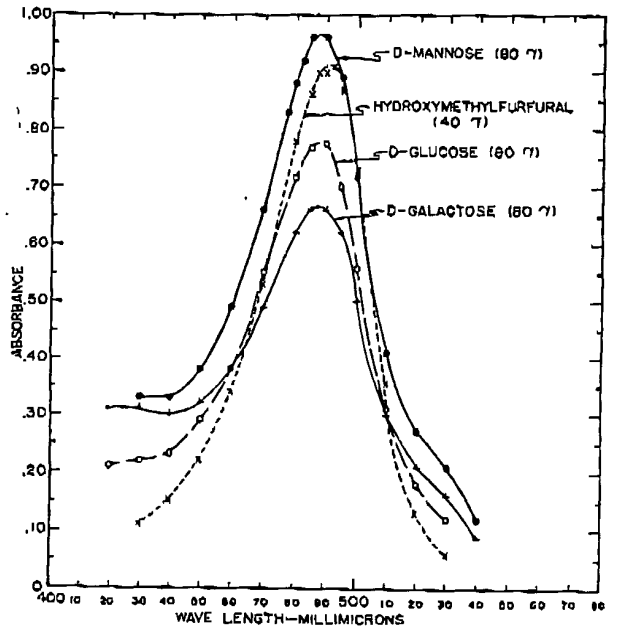

Figure 3. Absorption curves

Fast-delivery 5-ml. pipet, to deliver 5 ml. of concentrated sulfuric acid in 10 to 20 seconds. This is easily prepared by cutting a portion of the tip of a standard 5-ml. pipet.

Series of matched colorimetric tubes, internal diameter between 16 and 20 mm. This diameter will allow good mixing without dissipating the heat too rapidly. A high maximum temperature is desired because it increases the sensitivity of the reagent.

Series of micropipets delivering 0.02, 0.05, and 0.1 ml. The type described by Pregl (*41*) is satisfactory.

Procedure. Two milliliters of sugar solution containing between 10 and 70 γ of sugar is pipetted into a colorimetric tube, and 0.05 ml. of 80% phenol (adjust amount according to Figures 9 and 10) is added. Then 5 ml. of concentrated sulfuric acid is added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes are allowed to stand 10 minutes, then they are shaken and placed for 10 to 20 minutes in a water bath at 25° to 30° C. before readings are taken. The color is stable for several hours and readings may be made later if necessary. The absorbance of the characteristic yellow-orange color is measured at 490 mμ for hexoses and 480 mμ for pentoses and uronic acids. Blanks are prepared by substituting distilled water for the sugar solution. The amount of sugar may then be determined by reference to a standard curve previously constructed for the particular sugar under examination.

All solutions are prepared in triplicate to minimize errors resulting from accidental contamination with cellulose lint.

If it is desired to avoid the use of micropipets, the phenol may be added as a 5% solution in water. The amounts of reactants are then: 1 or 2 ml. of sugar solution, 1 ml. of 5% phenol in water, and 5 ml. of concentrated sulfuric acid. All other steps are the same as above.

Standard Curves. A series of typical standard curves is shown in Figures 1 and 2. Included in these figures are examples of some of the sugars usually encountered in carbohydrate studies—namely, pentose, deoxypentose, methylpentose, aldohexose, ketohexose, hexuronic acid, disaccharide, trisaccharide, and certain methylated derivatives. In order to test the method, the experiments were repeated on different days and by different operators. In all cases the variations between experiments and between operators were no more than 0.01 to 0.02 unit in absorbance, which was the same order of magnitude as the variation between the triplicate samples.

The experimental data for the various carbohydrates, except 2-deoxyribose, given in Figures 1 and 2 may be tabulated by calculating the value of a_s, the absorbance index, in the equation $A_s = a_s bc$ (Table I). The absorbance, A_s, is a dimensionless ratio equal to $\log_{10} \frac{T_{solvent}}{T_{solution}}$, where T is per cent transmittance, b is the length of light path, expressed in centimeters, and c is the concentration, in micrograms of sugar per milliliter of final volume.

Discussion of Results. ABSORPTION CURVES. The curves obtained by plotting absorbance *vs.* wave length (Beckman Model

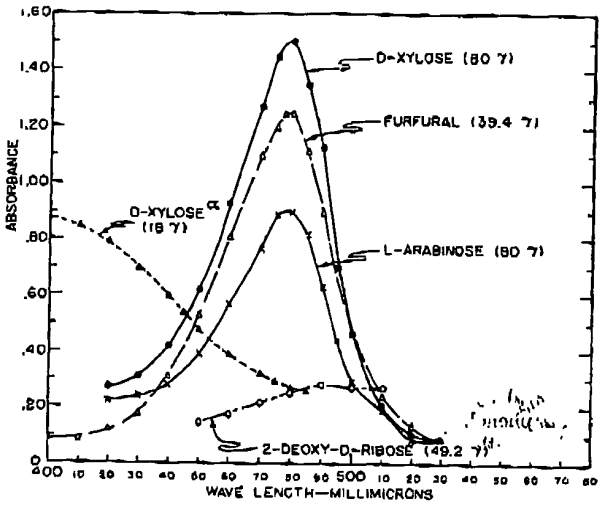

Figure 4. Absorption curves

A 0.1 ml. of butanol-ethanol-water chromatographic developing solvent (4 to 1 to 5, upper layer) was added in addition to the phenol

DU) are shown in Figures 3 to 8; the absorption curve is characteristic for each of the sugars described (*9*, *25*). The pentoses, methylpentoses, and uronic acids have an absorption maximum at 480 mμ, while hexoses and their methylated derivatives have an absorption maximum at 485 to 490 mμ. Certain of the methylated pentose sugars and their methyl glycosides show selective absorption at about 415 to 420 mμ (Figure 8) and for this reason the colorimetric determination of 2,3,5-tri-o-methyl-L-arabinose and its methyl glycoside is best carried out at 415 mμ.

The D-xylose and furfural curves are very similar. Assuming that the amount of color is proportional to the amount of furfural present or produced, the conversion of D-xylose to furfural under the conditions of the test is 93% of theory.

Calculation of conversion of D-xylose to furfural

	M.W.	Micrograms	Absorbance
Furfural	96	39.46	1.25
D-Xylose	150	80	1.50

The percentage, P, of xylose converted to furfural in the reaction as measured by the intensity of color developed can be calculated as illustrated below:

$$P = \frac{1.50}{1.25} \times \frac{39.46}{96} \times \frac{150}{80} \times 100 = 92.5\%$$

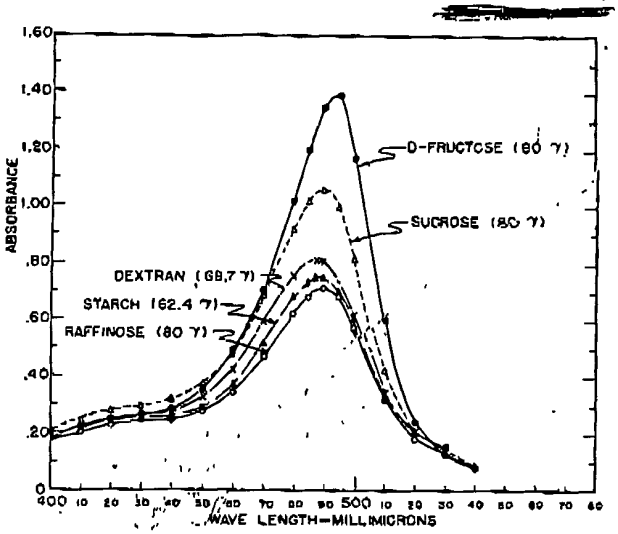

Figure 5. Absorption curves

Table I. Absorption Data for Certain Carbohydrates Determined by Phenol–Sulfuric Acid Reagent

Compound	Wt., γ	Phenol, Mg.[c]	Vol., Ml.	Light Path, Cm.	Instrument[a]	Wave Length, Mμ	Absorbance	a_s	Klett Reading[b]
D-Fructose	37.4	40	6.60	1	B	490	0.31	0.0547	
	42.4	51.6	6.01	1	B	490	0.35	0.0545	
	42.4	103	6.64	1	B	490	0.48	0.0752	
	42.4	154	6.68	1	B	490	0.52	0.0819	
	42.4	206	6.72	1	B	490	0.47	0.0902	
	42.4	310	6.80	1	B	490	0.68	0.0928	
D-Glucose	42.2	51.6	6.61	1	B	485	0.45	0.0704	
	42.2	103	6.64	1	B	485	0.448	0.0702	
	42.2	154	6.68	1	B	486	0.40	0.0632	
	80	40	6.60	1	B	487	0.78	0.0040	
Sucrose	53.6	40	6.60	1	B	490	0.48	0.0501	
	20.3	40	6.60	1	B	490	0.237	0.0504	
	85	100	6.64	1.6	C	490	0.395	0.0468	
5-Hydroxymethyl-2-furaldehyde	40	154	6.68	1	B	490	0.86	0.143	
	40	206	6.72	1	B	490	0.95	0.159	
	40	257	6.76	1	B	490	0.98	0.166	
Starch	62.4	103	6.64	1.27	K	Blue, No. 42	0.0328	0.00275	16.4
	124.8	103	6.64	1.27	K	Blue, No. 42	0.064	0.00268	32
	187.2	103	6.64	1.27	K	Blue, No. 42	0.096	0.00268	48
	312.0	103	6.64	1.27	K	Blue, No. 42	0.140	0.00236	72
	62.4	103	6.64	1	B	488	0.75	0.0799	
	124.8	103	6.64	1	B	488	1.46	0.0772	
Dextran	34.36	103	6.64	1.27	K	Blue, No. 42	0.0166	0.00252	8.3
	68.72	103	6.64	1.27	K	Blue, No. 42	0.0338	0.00257	16.9
	137.44	103	6.64	1.27	K	Blue, No. 42	0.0646	0.00240	32.3
	280.4	103	6.64	1.27	K	Blue, No. 42	0.1390	0.00252	69
	34.36	103	6.64	1	B	488	0.40	0.0774	
	68.72	103	6.64	1	B	488	0.81	0.0764	
D-Galacturonic acid	80	16	6.56	1.00	B	480	0.532	0.0430	
D-Mannuronc	80	40	6.60	1.00	B	485	0.39	0.0822	
D-Glucuronc	80	40	6.60	1.00	B	480	0.287	0.0237	
D-Galactose	80.2	40	6.60	1.00	B	487	0.664	0.0546	
D-Mannose	80	40	6.60	1.00	B	487	1.01	0.0830	
L-Arabinose	80	40	6.60	1.00	B	480	0.90	0.0742	
D-Xylose	80	40	6.60	1.00	B	480	1.50	0.1239	
L-Rhamnose	80	16	6.56	1.00	B	480	0.82	0.0674	
L-Fucose	80	16	6.56	1.00	B	480	0.35	0.0288	
Maltose	40	100	6.63	1.6	C	490	0.47	0.0402	
Raffinose	50	100	6.63	1.6	C	490	0.46	0.0381	
Lactose	50	100	6.63	1.6	C	490	0.355	0.0294	
2-o-Methyl-D-xylose	50	20	7.45	1.6	C	485	0.91	0.0289	
2,3-Di-o-methyl-D-xylose	58.6	20	7.45	1.6	C	480	0.39	0.031	
Methyl 2,3-di-o-methyl-D-xyloside	47.7	35	7.45	1.00	B	480	0.23	0.036	
Methyl 2,3-di-o-methyl-D-xyloside	47.7	35	7.45	1.00	B	415	0.21	0.0328	
3,3,5-Tri-o-methyl-L-arabinose	40	50	7.45	1.6	C	415	0.27	0.0314	
Methyl 2,3,5-tri-o-methyl-L-arabinoside	50	50	7.45	1.6	C	415	0.325	0.0302	
2,3-Di-o-methyl-D-glucose	80	40	6.60	1.00	B	485	0.76	0.0708	
2,3,6-Tri-o-methyl-D-glucose	53	40	6.60	1.00	B	485	0.555	0.0690	
2,3,4,6-Tetra-o-methyl-D-glucose	80	120	6.65	1.00	B	485	0.57	0.0474	
2,3-Di-o-methyl-D-mannose	50	50	6.57	1.6	C	485	0.39	0.0320	
2,3,6-Tri-o-methyl-D-mannose	50	50	6.57	1.6	C	485	0.37	0.0304	
2,3,4,6-Tetra-o-methyl-D-galactose	50	50	6.57	1.6	C	485	0.37	0.0304	

[a] B, Beckman Model DU; C, Coleman Junior; K, Klett-Summerson.

[b] Klett reading = $\frac{1000 \times \text{absorbance}}{2}$

[c] Actual weight of phenol. To find weight of 80% solution, divide by 0.8.

Calculation of final volume

2 ml. water	2
5 ml. sulfuric acid × 1.84	9.20
Total wt.	11.20 grams

Concn. of sulfuric acid after mixing $\frac{9.20 \times 0.95}{11.20} = 78\%$

Density of 78% sulfuric acid (20° C.) 1.7043

Volume of mixture $\frac{11.20}{1.70} = 6.57$ ml.

The addition of small amounts of phenol was considered to have a negligible effect on the density of the solution; hence, 0.1 ml. of 80% phenol would increase the volume by 0.06 ml.

2 ml. water	2
1 ml. 5% phenol in water	1
5 ml. sulfuric acid	9.2
Total wt.	12.2 grams

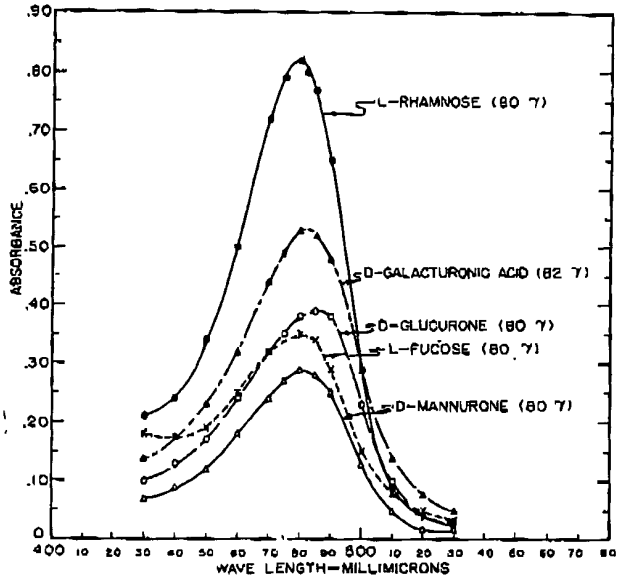

Figure 6. Absorption curves

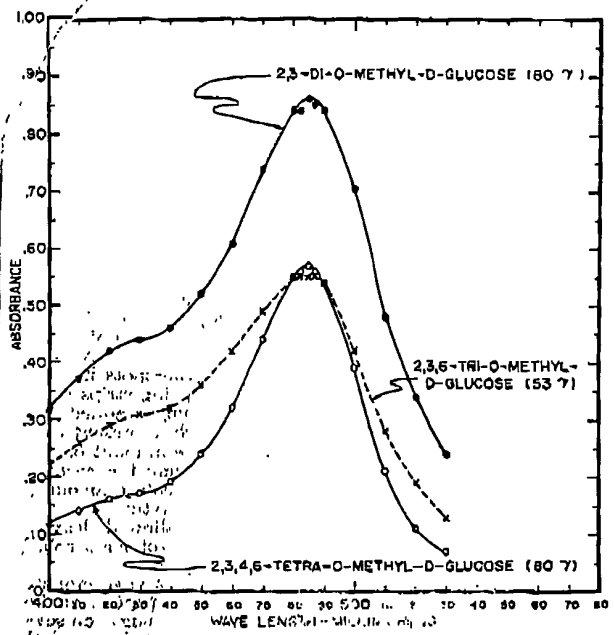

Figure 7. Absorption curves

Table II. Relationship between Index of Absorbance and Sugar Concentration as Determined by Different Instruments

Instrument	Approx. Band Width, Mμ	D-Mannose, γ	Light Path, Cm.	Absorbance	a_s
Beckman Model DU	0.5	80	1.00	1.01	0.0835
	0.5	40	1.00	0.495	0.0816
	0.5	20	1.00	0.25	0.0826
Coleman Jr.	60	41.1	1.6	0.45	0.0451
	60	20.5	1.0	0.24	0.0481
	60	10.2	1.6	0.11	0.0442
Evelyn	65	40	1.8	0.49	0.0426
	65	20	1.9	0.27	0.0464
	65	10	1.9	0.13	0.0473

Concn. of sulfuric acid $\frac{9.20 \times 0.95}{12.2} = 71.6\%$

Density at 20° C. 1.628

Volume of mixture $\frac{12.20}{1.628} = 7.48$ ml.

Effect of Variable Amounts of Phenol. The intensity of the color is a function of the amount of phenol added. As the amount of phenol is increased, the absorbance increases to a maximum and then usually falls off (Figures 9 and 10). When a paper chromatographic separation has been effected using phenol as a solvent, it will be found impractical to remove all of the phenol developer by air drying. This is not essential, though, because the curve of absorbance *vs.* amount of phenol is relatively flat after the maximum color intensity has been reached. Reproducible results can be obtained by operating at either side of the peak or at the peak as long as the amount of phenol added is controlled. This could conceivably form the basis for the analysis of mixtures of sugars—for instance, of D-mannose and D-glucose—by making two series of experiments, one at low and one at high phenol concentrations. The difference in readings is not large enough by itself except for rather crude estimations, but in combination with the variation in wave length of absorption maxima peaks between pentoses or uronic acids and hexoses, a satisfactory analysis might be devised.

A procedure using a somewhat similar idea, the rate of color development between sugars and the anthrone reagent, has been reported by Koehler (*28*).

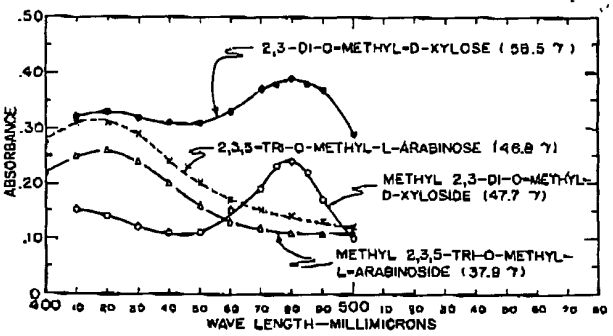

Figure 8. Absorption curves

Effect of Band Width. The absorbance, as is generally true in colorimetric determinations, is a function of the length of light path as well as the band width of the light source. As the band width becomes narrower, the observed absorbance becomes greater. If the values of the constant a_s are calculated from the equation $A_s = a_s bc$, the effect of the band width becomes apparent (Table II).

The higher the value of a_s, the more sensitive is the instrument. On this basis, the Beckman was the most sensitive instrument used; the others, however, perform well enough for routine analysis.

In the case of the Evelyn and the Coleman colorimeters, the value of a_s is not constant. This means that the plot of concentration *vs.* absorbance is not linear at the higher concentrations; however, it is very nearly linear at lower concentrations. The linearity of the plot of absorbance *vs.* concentration is extended to higher regions of concentration by operating at narrower band widths. The points obtained in the nonlinear region with the colorimeters passing wider bands are, nevertheless, reproducible (Table II).

Accuracy of Method. Under the proper conditions, the method can be expected to be accurate to within ±2%. This figure was obtained by plotting the results obtained by use of the Beckman Model DU spectrophotometer and comparing the amount of sugar actually present with that indicated by the plot. As mentioned previously, the narrow band width of the Beckman spectrophotometer makes it possible to extend the linearity of the standard curve. The percentage error is shown in Table III.

Table III. Accuracy of Phenol–Sulfuric Acid Method for Sugar Determination

Compound	Taken, γ	Found, γ	Error, %	Absorbance
Mannose	80	81	1.3	1.01
	40	39	2.5	0.495
	20	20	0.0	0.25
Galactose	80.4	79.5	1.1	0.665
	40.2	39.5	1.7	0.325
	21.4	21.5	0.5	0.175

Conclusions. The phenol–sulfuric acid method can be used to give reliable estimations of the sugar content of pure solutions. The colors produced are unusually stable, and possess a definite absorption peak. The amount of color produced at a constant phenol concentration is proportional to the amount of sugar present. The standard curves obtained by plotting the sugar concentration *vs.* the absorbance can be readily reproduced and, because of this, only one standard curve need be prepared for a given sugar. Furthermore, the reagents are inexpensive, stable, and readily available.

QUANTITATIVE ANALYSIS OF SUGARS BY PAPER CHROMATOGRAPHY

The application of qualitative paper chromatography to the separation of sugar mixtures has been extended to the field of quantitative analysis. Any sugars that can be separated by the technique of paper chromatography can be determined quantitatively by the colorimetric technique just described after elution from the paper (*13, 15, 29*). The principle is simple, but certain factors complicate the analysis. Probably the most serious of these is that carbohydrate impurities are extracted from the paper along with the sugar to be analyzed. This source of error is reduced greatly by the simple expedient of running a blank. The size of the blank reading may be reduced to about one half by washing the papers with distilled water containing about 1% ammonia (*37*). Another complicating factor is the introduction of cellulose lint during the elution procedure, but this can be eliminated entirely by careful filtration.

A procedure similar to the one described herein is reported by Dimler and others (*15*). However, their elution procedure is considerably more complicated than the one used in this work. Furthermore, the best colorimetric technique at the disposal of these workers was the anthrone method, the disadvantages of which have already been explained.

Washing of Paper. The following experiment illustrates how the soluble carbohydrate fraction present in filter paper may be reduced by washing. This fraction cannot be entirely washed out (*24*), and seems to increase after the washed paper is allowed to dry (*40*). Other work (*24*) in this laboratory has shown that the soluble carbohydrate fraction of filter paper is of the nature of a pentosan. The further study of this carbohydrate material will form the subject of another communication.

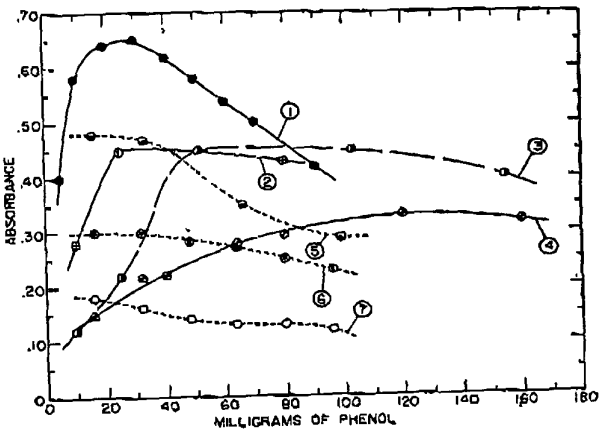

Figure 9. Absorbance *vs.* amount of phenol

1. D-Xylose, 40 γ, Coleman Jr., 480 mμ
2. D-Mannose, 41 γ, Evelyn, filter No. 490
3. D-Glucose, 42 γ, Beckman Model DU, 485 mμ
4. 2-Deoxy-D-ribose, 49 γ, Coleman Jr., 490 mμ
5. D-Galactose, 54 γ, Coleman Jr., 490 mμ
6. L-Rhamnose, 52 γ, Evelyn, filter No. 490
7. L-Fucose, 25 γ, Coleman Jr., 480 mμ

A piece of Whatman No. 1 filter paper 22 × 4 inches was washed with distilled water containing 0.5% ammonia and dried for 24 hours. The paper was added to a beaker containing 20 ml of distilled water and allowed to stand with occasional shaking for 20 minutes. The solution was filtered through a plug of glass wool and a 2-ml. aliquot of it was transferred to a colorimeter tube. Forty milligrams of phenol was added as an 80% solution of phenol in water and then 5 ml. of concentrated sulfuric acid. The absorbance of the solution was determined with an Evelyn colorimeter.

The solutions from the washed and unwashed papers showed absorbances of 0.03 and 0.06, respectively.

Procedure. Two sheets of Whatman No. 1 filter paper 8 × 22 inches are prepared as described below. One of the sheets is used as a blank. Before placing any sugars on the paper, lines are drawn as follows: Two lines are drawn lengthwise 1.5 inches from the edge of the paper. Two more lines are drawn, the first 1 inch from the top and the second 3.5 inches from the top. The sugars to be analyzed are placed on the paper along the 3.5-inch line. The two strips 1.5 inches from the edge are marking strips, which will be cut off and sprayed with *p*-anisidine or *p*-phenetidine trichloroacetate or ammoniacal silver nitrate after development of the chromatogram. The appearance of the spots marks the distance the sugars have traveled in the marking strips and the unsprayed center section. The amount of sugar added to the marking strip is not critical as long as enough is present to give a spot with the spray reagent. However, the amount of sugars added to the 5-inch center section of the paper must be accurately measured if it is desired to determine the absolute amounts of sugars as well as the relative amounts in the mixture.

A margin of at least 0.5 inch should be allowed, leaving 4 inches in the center to which a measured amount of sugar solution can be added from a micropipet.

The amount of sugar which can be added before overlapping of the spots occurs should be determined for each type of analysis. This can be done by putting graded amounts of sugar on several papers, drying, then developing with solvent, drying, and then spraying the entire paper. This will show whether the sugars

move in discrete bands, and how much margin should be allowed along the edges. The larger the amount of sugars which can be added, the less significance the blank will have. In most cases about 600 to 1000 γ of sugar should be added. Dimler and others (*15*) recommend that another paper be prepared to counteract the variations in delivery that may occur with micropipets. To this paper they add standard amounts of known sugars, using the same pipet and the same technique. This procedure does not, of course, eliminate the need for a blank determination, because the presence of the soluble carbohydrate fraction in the filter paper will have a relatively greater effect at low sugar concentrations. After the sugars have been added to the paper, the chromatograms are developed for a long enough period so that the sugars to be analyzed are clearly separated. After the chromatogram has been dried in the air, the side marking strips are cut off and sprayed to show the location of the sugars in the center section. The center unsprayed portion of the chromatogram is then cut up into sections corresponding to the locations of the sugar. Each section is transferred to Petri dishes, beakers, or other suitable containers that can be covered or closed. The blank paper is cut up to correspond to the area and location of the sugars of the other paper. Twenty milliliters of distilled water is added to each of the Petri dishes, which are then covered and allowed to stand for 30 minutes with occasional shaking. During this time the sugar becomes equally distributed throughout the liquid and solid phases (water and cellulose). The eluate is filtered through glass wool and the concentration of sugars determined as described before, with the important difference that the absorbance of the blank reading is subtracted from that corresponding to the sugar before referring to the standard curve.

Results. Efficiency of Extraction of Sugars from Filter Paper. This is illustrated by two typical experiments:

1. With a micropipet, 0.102 ml. of a solution containing 4.52 mg. of D-fructose was added to a piece of Whatman No. 1 paper (3 × 5 inches). The paper was allowed to dry in the air for 24 hours and then soaked in 20 ml. of distilled water for 0.5 hour to extract the sugar. (In another series of experiments it was found that sugars are extracted from the paper almost immediately.) The extract was filtered through glass wool and a 2-ml. aliquot of the filtrate added to 20 ml. of water. Two milliliters of this diluted solution was treated with 258.4 μl. of 80% aqueous phenol, followed by 5 ml. of concentrated sulfuric acid. The observed absorbance at 490 mμ was 0.545 and 0.538.

In a blank experiment a piece of paper of identical size was extracted for 0.5 hour with 20 ml. of water. A 2-ml. aliquot was treated with phenol and sulfuric acid as described above. The absorbance was 0.10 (average of three results). Hence, the absorbance correction for the blank = $0.10 \times \frac{2}{22} = 0.01$.

Corrected absorbance for the sugar determination = 0.54 − 0.01 = 0.53. From the standard curve for fructose, an absorbance of 0.57 is equivalent to 42.4 γ of sugar. Therefore, the amount of fructose equivalent to an absorbance of 0.53 = $\frac{0.53}{0.57}$ × 42.4 γ. Hence the total fructose recovered = $\frac{0.53}{0.57} \times 42.4 \times \frac{20}{2} \times \frac{22}{2} = 4336$ γ.

$$\text{Recovery} = \frac{4336}{4520} \times 100 = 96\%.$$

2. A similar experiment carried out with D-glucose (400 γ) added to a piece of paper (2 × 2 inches) gave a recovery of 100%. Additional experiments with D-mannose, D-xylose, and L-arabinose, and with methylated sugars such as 2,3,4,6-tetra-*o*-methyl-, 2,3,6-tri-*o*-methyl-, and 2,3-di-*o*-methyl-D-glucose with and without solvent migration using phenol-water, butanol-ethanol-water, and methyl ethyl ketone–water azeotrope gave recoveries of 95 to 100%.

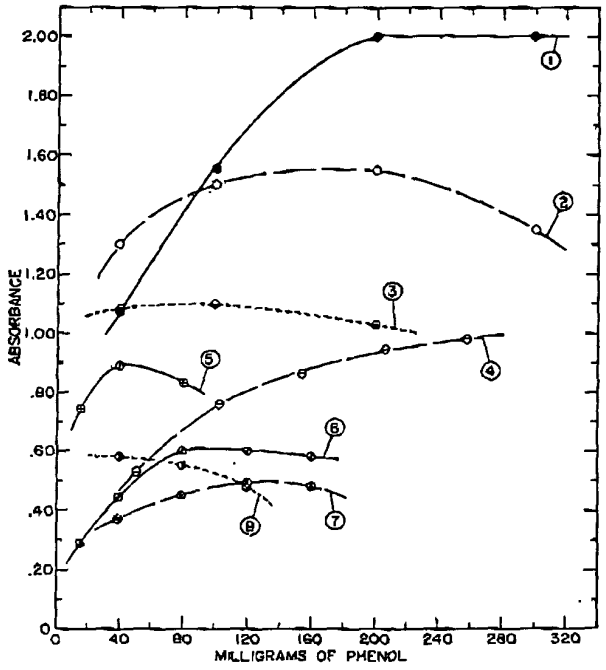

Figure 10. Absorbance *vs.* amount of phenol

1. D-Fructose, 80 γ, Beckman Model DU, 490 mμ
2. Sucrose, 80 γ, Beckman Model DU, 490 mμ
3. Raffinose, 80 γ, Beckman Model DU at 490 mμ
4. 5-(4 rietroxymethyl)-2-furaldehyde, 40 γ, Beckman Model DU, 485 mμ
5. Glucialo-methyl-D-glucose, 80 γ, Evelyn, filter No. 490
100 ml. of Idehyde, 20 γ, Evelyn, filter No. 490
Tetra-*o*-methyl-D-glucose, 80 γ, Evelyn, filter No. 490
——methyl-D-glucose, 51 γ, Evelyn, filter No. 490

Analysis of a Synthetic Mixture of Sugars. (1) A solution containing D-fructose (3.18 mg.) and D-glucose (0.20 mg.) was transferred to a piece of Whatman No. 1 paper (8 × 22 inches) as described previously. The chromatogram was developed for 24 hours by use of phenol saturated with water as the solvent. The paper was removed from the chromatographic chamber and allowed to dry for 24 hours. The marginal strips were cut off and sprayed with *p*-anisidine trichloroacetic acid reagent (small amounts of phenol do not interfere). After reassembling the chromatogram, the best line of demarcation was drawn between the two spots and the sections were cut out (glucose, 6 to 8.5 inches, fructose, 8.5 to 11 inches from the starting line), together with the corresponding blanks as previously described. The pieces of paper containing the two sugars and the two blanks were extracted and filtered. The concentration of the two sugars was then determined by the phenol–sulfuric acid reagent, reference being made to standard curves for glucose and fructose. The results were as follows:

Glucose Recovery

Absorbance of the eluate (2 ml. out of 20 ml. removed for test)	0.32
Absorbance of blank	0.10
Absorbance for glucose	0.22

From the standard curve for glucose absorbance, 0.45 = 42.4 γ glucose

Absorbance of 0.22 = $\frac{0.22}{0.45} \times 42.4$ γ glucose

Total glucose recovered = $\frac{0.22}{0.45} \times 42.4 \times \frac{20}{2} = 206$ γ glucose

Recovery = 103%.

Fructose Recovery

Absorbance of eluate (diluted 2 ml. to 20 ml. of water)	0.40
Absorbance of blank	0.01
Absorbance for fructose	0.39

From the standard curve for fructose absorbance, 0.57 = 42.4 γ fructose

Absorbance of 0.39 = $\frac{0.39}{0.57} \times 42.4$ γ fructose

Total fructose recovered = $\frac{0.39}{0.57} \times 42.4 \times \frac{20}{2} \times \frac{22}{2}$ = 3200 γ fructose
Recovery = 101%.

(2) For a solution containing D-mannose and D-glucose, the following results were obtained:

Solvent developer	1-butanol-ethanol-water
Time, hours	48
Paper	Whatman No. 3
D-Mannose added, γ	440
D-Mannose recovered, γ	417
% recovery	95
D-Glucose added, γ	470
D-Glucose recovered, γ	440
% recovery	93.5
Glucose in original mixture, %	51.5
Glucose calculated from analysis, %	51.3

The close agreement is fortuitous, but numerous experiments with mixtures of methylated and unmethylated sugars have shown that recoveries of 100 ± 5% or better are to be expected. In the above experiment the recoveries were not so good as expected, but it is believed that this is due to the fact that the sugar bands with Whatman No. 3 are less compact than those with Whatman No. 1; for this reason the No. 1 paper is preferred.

Table IV. Wave Length *Vs.* Absorbance for Starch[a]

(Starch-phenol-sulfuric acid, Beckman Model DU, slit width 0.1 mm., 103 mg. of phenol)

Wave Length	Absorbance for 62.4 γ Starch	Absorbance for 124.8 γ Starch
410	0.21	0.42
420	0.24	0.47
430	0.25	0.495
440	0.257	0.51
450	0.29	0.578
460	0.371	0.745
470	0.52	1.03
480	0.68	1.32
485	0.735	1.42
488	0.75	1.45
490	0.75	1.46
495	0.70	1.35
500	0.60	1.15
510	0.33	0.635
520	0.197	0.388
530	0.147	0.294

[a] Baker's potato starch dried for 3 days in vacuo (30 mm.) at 75° C.

Conclusions. The phenol–sulfuric acid method can be applied to the analysis of any mixtures of sugars and their methyl derivatives that are amenable to separation by paper chromatography. Thus it has been applied to the analysis of mixtures of methyl sugars separated on paper by butanol-ethanol-water or methyl ethyl ketone–water azeotrope. The method has also proved of value for the analysis of hydrolyzates of oligosaccharides; of polysaccharides such as starch (Table IV), glycogen, plant gums, and hemicelluloses (*15*); and for the determination of the amount of sugar in urine and in blood.

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RECEIVED for review June 24, 1955. Accepted December 28, 1955.

Digester and Filter for Preparing Extract Solutions from Solids—Correction

After publication of the article on "Digester and Filter for Preparing Extract Solutions from Solids" [ANAL. CHEM. 27, 1669 (1955)] attention was called to an article published a short time earlier by M. Potterat and H. Eschmann [*Mitt. Lebensm. Hyg.* 45, 329–31 (1954)], in which a design for an apparatus having substantially the same features was presented. Since receiving this information the authors have sought to learn how the earlier article escaped notice and found that because of the time factor the publication in which it appeared could not have been available to them when the manuscript was prepared.

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155
27
179
127
206

THIS OPINION WAS NOT WRITTEN FOR PUBLICATION

The opinion in support of the decision being entered today (1) was not written for publication in a law journal and (2) is not binding precedent of the Board.

Paper No. 152

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

RICHARD G.F. VISSER; EVERT JACOBSEN; and WILLEM J. FEENSTRA

Junior Party,

v.

PER HOFVANDER; PER T. PERSSON; ANNELI TALLBERG, deceased, by LENNART HANSSON, Legal Representative; and OLLE WIKSTROM

Senior Party.

Interference 103,579

Final Hearing: July 18, 2001

Before METZ, GRON, and LORIN Administrative Patent Judges.

GRON, Administrative Patent Judge.

Interference 103,579

FINAL DECISION

1. Background

December 21, 1990 - AMYLOGENE HB c/o Svalof AB filed Swedish Patent Application 9004096-5 for "Genetically Engineered Modification of Potato to Form Amylopectin-Type Starch" (hereafter Hofvander's Swedish application).

December 20, 1991 - PER HOFVANDER, PER T. PERSSON, ANNELI TALLBERG, and OLLE WIKSTROM filed PCT International Application PCT/SE91/00892, for "Genetically Engineered Modification of Potato to Form Amylopectin-Type Starch" (hereafter Hofvander's PCT application), claiming benefit of the December 21, 1990, filing date of Hofvander's Swedish application.

February 14, 1992 - RICHARD G.F. VISSER, EVERT JACOBSEN, and WILLEM J. FEENSTRA filed U.S. Application 07/835,886, for "Potato Plant Producing Essentially Amylose-Free Starch" (hereafter Visser's grandparent application).

July 9, 1992 - International Publication Number WO92/11376 (hereafter Hofvander's PCT publication) issued from Hofvander's PCT application filed December 20, 1991.

November 24, 1993 - PER HOFVANDER; PER T. PERSSON; ANNELI TALLBERG, deceased, by LENNART HANSSON, Legal Representative; and OLLE WIKSTROM filed involved U.S. Application 08/070,455, for "Genetically Engineered Modification of Potato to Form Amylopectin-Type Starch" (hereafter Hofvander's involved

application (Hofvander's Record, pages 275-316 (HR 275-316))) claiming benefit of the December 20, 1991, filing date of Hofvander's PCT application, and the December 21, 1990, filing date of Hofvander's Swedish application.

December 1, 1993 - Visser filed U.S. Application 08/159,714 (hereafter Visser's parent application), as a divisional of Visser's grandparent application, filed February 14, 1992.

August 23, 1994 - Visser filed U.S. Application 08/294,619 (hereafter Visser's involved application (Visser's Record, pages 139-184 (VR 139-184))), as a continuation of Visser's parent application, filed December 1, 1993, which is a divisional of Visser's grandparent application, filed February 14, 1992.

April 5, 1996 - Interference 103,579 was declared essentially as follows (Paper No. 2):

Junior Party

Applicants:	Richard G.F. Visser, Evert Jacobsen, and Willem J. Feenstra
Serial No.:	08/294,619, August 23, 1994
Accorded Benefit:	U.S. Applications 08/159,714, filed December 1, 1993, and 07/835,886, filed February 14, 1992

Senior Party

Applicants:	Per Hofvander; Per T. Persson; Anneli Tallberg, deceased, by Lennart Hansson, Legal Representative; and Olle Wikstrom
Serial No.:	08/070,455, filed November 24, 1993

Interference 103,579

Accorded Benefit: International Application PCT/SE91/00892, filed December 20, 1991

Count 1

A homologous construct of the potato plant comprising a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA.

The claims of the parties which were designated as corresponding to Count 1 were:

Hofvander: Claims 1, 4, and 6-23

Visser: Claims 1, 4-8, 11, 13-20, and 23-27.

June 6, 1995 - Hofvander filed U.S. Application 08/470,720 (hereafter Hofvander's patented application) as a continuation of Hofvander's involved application, filed November 24, 1993, first filed December 20, 1991, as Hofvander's PCT application.

September 5, 1996 - Visser moved under 37 CFR § 1.633(b) for judgment that there is no interference in fact (Visser's Preliminary Motion 1 (Paper No. 17)) because none of Visser's claims designated as corresponding to Count 1 are directed to the same patentable invention as any of Hofvander's claims designated as corresponding to Count 1 (Paper No. 17, p. 2, para. 2).

September 5, 1996 - Visser moved under 37 CFR § 1.633(a) for judgment that Claims 1, 4, and 6 to 23 of Hofvander's involved application, filed November 24, 1993, designated as corresponding to Count 1, are unpatentable under 35 U.S.C. § 102 over Hergersberg, "A Molecular Analysis of the *waxy* Gene from Solanum tuberosum and Expression of *waxy* antisense RNA in

transgenic Potatoes," Inaugural-Dissertation zur Erlangung des Doktorgrades der Mathematisch-Naturwissenschaftlichen Fakultat der Universitat zu Koln, University of Cologne, Cologne, pp. 1-79 (1988)(Visser Documentary Exhibit 1 (Visser's Documentary Exhibit 1 (VDX 1)), or Hovenkamp-Hermelink, et al. (Hovenkamp-Hermelink), "Isolation of an Amylose-Free Starch Mutant of the Potato (Solanum tuberosum L.)," Theor. Appl. Genet., Vol. 75, pp. 217-221 (1987)(VDX 9); and/or under 35 U.S.C. § 103 in view of the combined teachings of Hergersberg; Hovenkamp-Hermelink; Visser (Visser's PhD Thesis), "Manipulation of the Starch Composition of Solanum Tuberosum L. Using *Agrobacterium Rhizogenes* Mediated Transformation," PhD Thesis, University of Groningen, The Netherlands, pp. 9-139 (February 27, 1989)(VDX 7); and van der Leij et al. (van der Leij), "Sequence of the Structural Gene for Granule-Bound Starch Synthase of Potato (Solanum tuberosum L.) and Evidence for a Single Point Deletion in the *amf* Allele," Mol. Gen. Genet., Vol. 228, pp. 240-248 (1991)(VDX 3)(Visser's Preliminary Motion No. 2 (Paper No. 18)).

September 5, 1996 - Visser moved under 37 CFR § 1.633(a) for judgment that Hofvander's Claims 1, 4, 6-20, and 22 are unpatentable under 35 U.S.C. § 112, first paragraph (Visser's Preliminary Motion No. 3 (Paper No. 19)).

September 5, 1996 - Visser moved under 37 CFR § 1.633(a)

for judgment that Hofvander's Claims 1, 4, and 6-23 are unpatentable under 35 U.S.C. § 102 over Visser et al. (Visser's 1991 publication), "Inhibition of the Expression of the Gene for Granule-Bound Starch Synthase in Potato by Antisense Constructs," Mol. Gen. Genet., Vol. 225, pp. 289-296 (1991)(VDX 8)(Visser's Preliminary Motion No. 4 (Paper No. 20), contingent on denial of Visser's Preliminary Motion No. 1 (Paper No. 17)).

September 5, 1996 - Visser moved under 37 CFR § 1.633(c)(4) to have Visser's Claims 1, 4, 8, 11, 13-20, 22, and 24-27 designated as not corresponding to Count 1 (Visser's Preliminary Motion No. 5 (Paper No. 21)).

September 5, 1996 - Contingent upon denial of Visser Preliminary Motions 1-5, Visser moved under 37 CFR § 1.633(c)(1) to redefine the interfering subject matter by substituting a new Count V-1[1] for Count 1 (Visser's Contingent Preliminary Motion No. 6 (Paper No. 22)).

September 5, 1996 - Visser moved under 37 CFR § 1.633(f) to be accorded benefit of the filing dates of Visser's grandparent application, filed December 1, 1993, and Visser's parent application, filed February 14, 1992, for proposed Count V-1

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[1] Proposed Count V-1

A homologous construct of the potato plant comprising potato granule-bound starch synthase (PGBSS) genomic DNA oriented in the antisense direction.

(Visser's Preliminary Motion No. 7 (Paper No. 23)).

September 9, 1996 - Hofvander moved under 37 CFR § 1.633(c)(1) to substitute proposed Count H-1[2] or, in the alternative, proposed Count H-2[3] for Count 1 of the interference (Hofvander Preliminary Motion 1 (Paper No. 28)).

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[2] Proposed Count H-1

A homologous construct of the potato plant comprising a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA,

or

an antisense construct for inhibiting expression of the gene for granule-bound starch synthase in potato, comprising

a) a promoter, and
b) a fragment of the potato gene coding for granule-bound starch synthase inserted in the antisense direction, wherein said fragment has the amino acid of SEQ ID No. 1.

[3] Proposed Count H-2

An antisense construct for suppressing expression of the potato granule-bound starch synthase gene (GBSS gene) comprising

(a) a promoter, and
(b) a fragment of the potato GBSS gene inserted in the antisense direction, wherein said fragment is of sufficient length to result in the supression of amylose formation when introduced into the genome of a potato tissue and said potato is cultivated.

September 9, 1996 - Hofvander moved under 37 CFR § 1.633(c)(2) to redefine the interfering subject matter by amending claims designated as corresponding to the count and adding claims to be designated as corresponding to Proposed Count H-1 or H-2 (Hofvander Preliminary Motion 2 (Paper No. 29)).

September 9, 1996 - Hofvander moved under 37 CFR § 1.633(f) to be accorded benefit of the December 21, 1990, filing date of Hofvander's Swedish application and to be accorded benefit of the December 20, 1991, filing date of Hofvander's PCT application for Hofvander Proposed Count H-1 or, in the alternative, Proposed Count H-2 (Hofvander Preliminary Motion 3 (Paper No. 30)).

September 9, 1996 - Hofvander moved under 37 CFR § 1.633(c)(3) to have Visser's Claim 22 designated as corresponding to Count 1, Hofvander Proposed Count H-1, or, in the alternative, Hofvander Proposed Count H-2 (Hofvander Preliminary Motion 4 (Paper No. 31)).

September 25, 1996 - Hofvander moved under 37 CFR § 1.633(i) to redefine the interfering subject matter and amend its claims designated as corresponding to the count under 37 CFR § 1.633(c)(2)(Hofvander Preliminary Motion 4 (sic 5)(Paper No. 35)).

November 20, 1996 - Hofvander moved under 37 CFR § 1.635 for inter partes testing under 37 CFR § 1.639(g)(Hofvander Motion 5 (sic 6)(Paper No. 66)).

November 20, 1996 - Hofvander moved under 37 CFR § 1.635 to amend Hofvander's Proposed Count H-1[4] to correct an inadvertent error (Hofvander Motion 6 [sic 7](Paper No. 67)).

December 24, 1996 - An Administrative Patent Judge (APJ) decided the parties' preliminary motions as follows (Paper No. 74).

(1) Visser's Preliminary Motion 1 (VPM 1)(Paper No. 17) for judgment of no interference-in-fact was denied for the following reasons (Paper No. 74, pp. 4-5):

> A potato normally produces both amylose and amylopectin with the amylose normally being present in an amount of 20 to 25%. Both parties' claimed inventions are directed to improving the production of amylopectin in potatoes by the incorporation of antisense DNA matter into the potato plant genome.
>
> The Visser claimed invention is directed to the incorporation of the full length antisense potato granule bound starch synthase (PGBSS) cDNA or gDNA

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[4] Proposed Count H-1 (amended)

A homologous construct of the potato plant comprising a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA,

or

an antisense construct for inhibiting expression of the gene for granule-bound starch synthase in potato, comprising

a) a promoter, and
b) a fragment of the potato gene coding for granule-bound starch synthase inserted in the antisense direction, wherein said fragment has the nucleotide sequence of SEQ ID No. 1.

> into a potato plant, thereby inhibiting the production of amylose. According to the evidence relied upon by Visser, the Visser modified potato plants produce 100% amylopectin and inhibit the production of any amylose.
>
> The Hofvander claimed invention is directed to the incorporation of antisense fragments of the PGBSS gene into a potato plant to inhibit the production of amylose. According to the evidence relied upon by Hofvander, his modified potato plants produce 91 to 94% amylopectin, the remainder being amylose.
>
> The difference in amylopectin production by using Visser's modified potato plants rather than Hofvander's modified potato plants is about 6%. The Vissen [sic] evidence, however, does not show that the difference is unexpected. While Visser's potato plants and Hofvander's potato plants produce differing amounts of amylopectin, both sets of plants produce amylopectin in increased amounts over unmodified potato plants. The APJ agrees with the Hofvander opposition that Visser's motion fails to show that the difference in activity is unexpected thereby rendering the Visser claims unobvious. See, in general, In re Merck & Co., Inc., 800 F.2d 1091, 1099, 231 USPQ 375, 381 (Fed. Cir. 1986).

(2) Visser's Preliminary Motion 2 (VPM 2)(Paper No. 18) for judgment that Claims 1, 4, and 6 to 23 of Hofvander involved application, filed November 24, 1993, are unpatentable under 35 U.S.C. § 102 over Hergersberg (VDX 1)) or Hovenkamp-Hermelink (VDX 9); and/or under 35 U.S.C. § 103 in view of the combined teachings of Hergersberg, Hovenkamp-Hermelink, Visser's PhD Thesis (VDX 7) and van der Leij (VDX 3); was "denied for the reasons stated in Hofvander's opposition (Paper No. 47) with respect to Hofvander's claims 1, 4, and 7 to 23 and . . .

dismissed as moot with respect to claim 6" (Paper No. 74, p. 5)[5] with the following emphasis (Paper No. 74, pp. 5-6):

> With respect to the Hergersberg publication, the APJ agrees with Hofvander that this would not render the Hofvander claims unpatentable. The Hergersberg antisense sequences, assuming the sequences are antisense, are much smaller than those used by Hofvander. When the Hergersberg antisense sequences are incorporated into a potato plant, the modified potato plant reduced amylose production by 30%. Since a potato normally produces amylose in an amount of 20 to 25%, it would appear that Hergersberg's modified potato plants produced amylose in an amount of from 14% to 18%, whereas Hofvander's modified potato plants result in production of 6 to 9% amylose. Moreover, in distinguishing over the Hergersberg publication, the Hofvander opposition (pages 6 and 7) also relies upon the same reasons as did Visser in urging that his claims were unpatentable over this publication. Since an interference-in-fact exists between both parties' claims, the APJ is certainly persuaded by the foregoing argument that the Hofvander claims are also patentable over Hergersberg.

(3) Visser's Preliminary Motion 3 (VPM 3)(Paper No. 19) for judgment that Claims 1, 4, 6-20, and 22 of Hofvander's involved application, filed November 24, 1993, are unpatentable under 35 U.S.C. § 112, first paragraph, was dismissed as moot because Hofvander deleted the subject matter to which Visser objected (Paper No. 74, pp. 6-7).

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[5] The APJ stated that final judgment against Claim 6 would be entered because Hofvander attempted to cancel the claim (Paper No. 74, p. 5 n. 1).

(4) Visser's Preliminary Motion 4 (VPM 4)(Paper No. 20) for judgment that Claims 1, 4, and 6-23 of Hofvander's involved application, filed November 24, 1993, are unpatentable under 35 U.S.C. § 102 over Visser's 1991 publication (VDX 8), was granted (Paper No. 74, p. 7). However, the decision is based on a prima facie case of obviousness under 35 U.S.C. § 103 having been established in view of Visser's 1991 publication (Paper No. 74, p. 8):

> By opposing Visser's preliminary motion 1 for judgment on the ground of no interference-in-fact, Hofvander has conceded that Visser's claims, which are directed to introducing full length antisense cDNA PGBBS [sic, PGBSS] into a potato, render obvious Hofvander's claims which are directed to introducing antisense fragments of PGBBS [sic, PGBSS] into a potato. See also, the arguments made by Hofvander in his opposition (Paper No. 46) to the Visser motion (1) . . . which arguments the APJ relies upon to show obviousness.

Presuming that a prima facie case of obviousness under 35 U.S.C. § 103 is established in view of the disclosure of Visser's 1991 publication, it was further determined that Hofvander had not shown its entitlement to benefit under 35 U.S.C. § 119 of the filing date of Hofvander's Swedish application for the full scope of the subject matter claimed (Paper No. 74, pp. 8-9). In denying Hofvander's claim for priority under 35 U.S.C. § 119, the decision read (Paper No. 74, p. 9):

> It is evident . . . that the Swedish priority document contains a written description for the fragment having a nucleotide sequence of SEQ ID No. 1. Since the Hofvander claims embrace this fragment and other fragments, Hofvander is not entitled to the benefit of the Swedish priority document with respect to claims 1, 4, and 6 to 23. Judgment against these claims will be entered when a final judgment is entered in this case. The Swedish priority document only supports the full scope of claim 24 . . . .

(5) Hofvander's Preliminary Motions 1 (HPM 1)(Paper No. 28) and 7 (HPM 7)(Paper No. 67) to substitute Proposed Count H-1 (as amended) and 3 (HPM 3)(Paper No. 30) to accord Hofvander benefit of the December 21, 1990, foreign filing date of Hofvander's Swedish application for corrected Proposed Count H-1, were granted (Paper No. 74).

(6) Hofvander's Preliminary Motion 2 (HPM 2)(Paper No. 29) to add Claim 24 Hofvander's involved application, filed November 24, 1993, to the interference as renumbered Claim 50 (Paper No. 77) was granted (Paper No. 74, p. 11). The same motion to amend other claims and to add to the interference and designate Claims 25-49 of Hofvander's involved application as corresponding to the count without due explanation or reasons therefore was dismissed (37 CFR § 1.637(a))(Paper No. 74, p. 11).

(7) Hofvander's Preliminary Motion 4 (HPM 4) (Paper No. 31) to have Visser's Claim 22 designated as corresponding to Hofvander Proposed Count H-1, now Count 2,

to correct an apparent inadvertent error, was granted (Paper No. 74, p. 12).

(8) Hofvander's Preliminary Motion 5 (HPM 5)(Paper No. 35) to amend Claims 1, 4, 7, and 10 of Hofvander's involved application, filed November 24, 1993, by deleting the phrase "fragments encoding the amino acid sequences of SEQ ID Nos. 6-17" and redefine the interfering subject matter by designating the claims, as amended, as corresponding to the count, was granted (Paper No. 74, p. 12).

(9) Visser's Preliminary Motion 5 (VPM 5)(Paper No. 21) to have Visser's Claims 1, 4, 8, 11, 13-20, 22, and 24-27 designated not to correspond to the count was denied "for the reasons stated by Hofvander's opposition (Paper No. 50)" (Paper No. 74, pp. 12-13).

(10) Visser's Preliminary Notions 6 (VPM 6)(Paper No. 22) and 7 (VPM 7)(Paper No. 23) to redefine the interfering subject matter by substituting proposed Count V-1 for Count 1 and to be accorded benefit of the filing dates of Visser's grandparent application, filed December 1, 1993, and Visser's parent application, filed February 14, 1992, for proposed Count V-1, both motions contingent upon denial of Visser's Preliminary

Motions 1-5, were dismissed because Visser's Preliminary Motion 4 (Paper No. 20) was granted (Paper No. 74, p. 13).

(11) Hofvander's Preliminary Motion 6 (HPM 6)(Paper No. 66) for inter partes testing of the parties' starches by an independent laboratory was denied "for the reasons stated by Visser" (Paper No. 74, p. 13).

January 29, 1997 - The interference was redeclared with corrected Proposed Count H-1 (new Count 2) substituted for existing Count 1 (Paper No 83). The interference was redeclared with new Count 2 as follows (Paper No. 83 (VR vii; HR viii)):

COUNT 2

A homologous construct of the potato plant comprising a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA

or

an antisense construct for inhibiting expression of the gene for granule-bound starch synthase in potato, comprising

a) a promoter, and
b) a fragment of the potato gene coding for granule-bound starch synthase inserted in the antisense direction, wherein said fragment has the nucleotide sequence of SEQ ID No. 1.

The claims of the parties which correspond to this count are:

Hofvander, et al.: claims 1, 4, 6 to 23 and 50

Visser, et al.: claims 1, 4 to 8, 11, 13 to 20 and 22 to 27[.]

Hofvander's claims designated as corresponding to Count 2 are reproduced below:

1. A method of suppressing amylose formation in potato, wherein the potato is modified by genetic engineering, which method comprises cultivating a potato containing in the genome of a tissue of said potato a gene construct comprising a fragment of the potato gene which codes for formation of granule-bound starch synthase (GBSS gene) inserted in the anti-sense direction, wherein said fragment is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, together with a promoter selected from the group consisting of CAMV 35S, patatin I and the GBSS promoter.

4. A fragment of a potato gene coding for granule-bound starch synthase (GBSS), wherein said fragment is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3.

6. Isolated potato gene coding for granule-bound starch synthase in potato (GBSS gene) having the nucleotide sequence stated in SEQ ID No. 5.

7. An antisense construct for inhibiting expression of the potato gene which codes for granule-bound starch synthase (GBSS gene) comprising

a) a promoter,
b) a fragment of the potato gene coding for granule-bound starch synthase inserted in the antisense direction, wherein said fragment is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3.

8. Antisense construct as claimed in claim 7, characterized in that the promoter is an isolated promoter from the potato gene coding for granule-bound starch syntase (GBSS).

9. Antisense construct as claimed in claim 7, characterized in that the promoter is selected from the group consisting of the CaMV 35S promoter and the patatin I promoter.

10. A vector comprising a fragment of the potato gene coding for granule-bound starch synthase (GBSS), wherein

said fragment is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, and said fragment is inserted in the antisense direction in relation to a promoter immediately upstream from the gene fragment.

11. Vector comprising the antisense construct as claimed in claim 7.

12. Cell of potato plant whose genome comprises the antisense construct as claimed in claim 7.

13. Potato plant whose genome comprises the antisense construct as claimed in claim 7.

14. Potato tubers whose genome comprises the antisense construct as claimed in claim 7.

15. Seeds from potato plant, whose genome comprises the antisense construct as claimed in claim 7.

16. Microtubers of potato, whose genome comprises the antisense construct as claimed in claim 7.

17. Vector comprising the antisense construct as claimed in claim 8.

18. Cell of potato plant whose genome comprises the antisense construct as claimed in claim 8.

19. Potato plant whose genome comprises the antisense construct as claimed in claim 8.

20. Potato tubers whose genome comprises the antisense construct as claimed in claim 8.

21. A method for tuber-specific expression of a gene product in potato, comprising transforming said potato with a DNA molecule comprising an isolated promoter from the potato gene coding for granule-bound starch synthase (GBSS).

22. Antisense construct as claimed in claim 7, characterized in that the promoter has the sequence stated in SEQ ID No. 4.

23. A method for tuber-specific expression of a gene product in potato, comprising transforming said potato with

a DNA molecule comprising an isolated promotor [sic] from the potato gene coding for granule-bound starch synthase (GBSS), said promoter having the nucleotide sequence stated in SEQ ID No. 4.

50. A method of suppressing amylose formation in potato, wherein the potato is modified by genetic engineering, which method comprises cultivating a potato containing in the genome of a tissue of said potato a gene construct comprising a fragment of the potato gene which codes for formation of granule-bound starch synthase (GBSS gene) inserted in the anti-sense direction, wherein said fragment has the nucleotide sequence of SEQ ID No. 1.

Visser's claims designated as corresponding to Count 2 are reproduced below:

1. A transgenic potato plant which, as a result of genetic engineering has a genome containing at least one gene construct containing a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence coding for PGBSS in reverse orientation in an expression cassette which is functional in potato plants, said gene construct giving rise to tubers containing essentially amylose free starch; wherein said expression cassette comprises in the 5'-3' direction of transcription: an upstream promoter base sequence, a base sequence for transcription into mRNA under control of said upstream promoter base sequence comprising coding and template strands, and a downstream transcription terminator base sequence, wherein the coding strand of said base sequence for transcription comprises an inverted sequence of bases complementary to a run of bases of PGBSS mRNA, wherein the transcript of said base sequence for transcription substantially inhibits the expression of PGBSS.

4. The transgenic potato plant according to claim 1 wherein said upstream promoter sequence is the cauliflower mosaic virus 35S promoter (P_{CaMV}).

5. The transgenic potato plant according to claim 1 wherein said upstream promoter sequence is the PGBSS promoter.

6. The transgenic potato plant according to claim 1 wherein said terminator is nopaline synthase terminator (T_{nos}).

7. The transgenic potato plant according to claim 1 wherein said terminator is PGBSS terminator.

8. The potato plant of claim 1, wherein the gene construct contains the neomycin phosphotransferase II gene (NPT-II) kanamycin resistance marker.

11. A tuber of the potato plant of claim 1.

13. The transgenic potato plant of claim 1 wherein said construct contains full length PGBSS cDNA.

14. The transgenic potato plant of claim 1 wherein said base sequence for transcription comprises a sequence of bases complementary to the sequence as set forth in Figure 3.

15. A method for producing a transgenic potato plant exhibiting at least one modified phenotypic trait by inhibiting the expression of an endogenous gene, said method comprising:

integrating into the genome of the plant cell at least one gene construct containing a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence coding for PGBSS in reverse orientation in an expression cassette which is functional in potato plants comprising in the 5'-3' direction of transcription: an upstream promoter base sequence, a base sequence for transcription into mRNA under control of said upstream promoter base sequence comprising coding and template strands, and a downstream transcription terminator base sequence functional in said cell wherein a transformed cell is obtained; and growing said transformed plant cell, wherein the coding strand of said base sequence for transcription comprises an inverted sequence of bases complementary to a run of bases of PGBSS mRNA, wherein the transcript of said base sequence for transcription substantially inhibits the expression of potato granule-bound starch synthase.

16. The method according to claim 15 wherein said construct further comprises T-DNA.

17. The method according to claim 15 wherein upsteam promoter sequence is CaMV35S promoter.

18. The method according to claim 15 wherein upstream promoter sequence is PGBSS promoter.

19. The method according to claim 15 wherein said terminator is nopaline synthase terminator.

20. The method according to claim 15 wherein said terminator is potato granule-bound starch synthase terminator.

22. The method according to claim 15 wherein the gene construct was integrated into the potato genome by transformation with Agrobacterium selected from a group consisting of Agrobacterium rhizogenes and Agrobacterium tumefaciens.

23. A homologous construct of the potato plant comprising a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA.

24. The homologous construct according to claim 23 wherein the PGBSS cDNA or genomic DNA is in reverse orientation.

25. The transgenic potato plant according to claim 1 further comprising variable numbers of integrated gene construct.

26. The method according to claim 15, further comprising integrating variable numbers of gene construct in the transformed plants.

27. The method according to claim 26, wherein there is no correlation between the number of PGBSS genes integrated copies and phenotypic effect.

July 2, 1997 - Visser filed its first (Paper No. 116), second (Paper No. 117), third (Paper No. 118), and fourth (Paper No. 119) motions to suppress evidence.

July 3, 1997 - Visser filed the Opening Brief of Visser et al. (VB)(Paper No. 122).

August 27, 1997 - Hofvander filed a motion to suppress evidence (Paper No. 123).

August 27, 1997 - Hofvander filed the Main Brief at Final Hearing of Senior Party Hofvander et al (HB)(Paper No. 128).

October 6, 1997 - Visser filed the Reply Brief of Visser et al. (VRB)(Paper No. 137).

October 20, 1998 - U.S. Patent 5,824,798 (Paper No. 141), assigned to Amylogene HB, Svalov, Sweden, and naming Anneli Tallberg, Per Hofvander, Per T. Persson, and Olle Wikstrom as inventors (Hofvander's patent), issued from Hofvander's application, filed June 6, 1995. Hofvander's patent claims:

> (1) A process for producing an amylopectin-type starch comprising:
>
> obtaining a potato tissue which has been transformed by introducing into the genome of the potato tissue a gene construct comprising a promoter and a fragment of the potato gene which codes for the information of granule-bound starch synthase inserted in the anti-sense direction, wherein said fragment essentially has a nucleotide sequence which is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3;
>
> growing the transformed potato tissue to produce a potato plant containing potato tubers;
>
> producing at least one potato from said potato tubers; and separating starch from said potato, wherein said starch is an amylopectin-type starch which is essentially free of amylose.
>
> (2) The process for producing an amylopectin-type starch according to claim 1, wherein said fragment has a nucleotide sequence of SEQ ID No. 1.

(3) The process for producing an amylopectin-type starch according to claim 1, wherein said fragment has a nucleotide sequence of SEQ ID No. 2.

(4) The process for producing an amylopectin-type starch according to claim 1, wherein said fragment has a nucleotide sequence of SEQ ID No. 3.

(5) The process for producing an amylopectin-type starch according to claim 1, wherein said promoter comprises a CAMV 35S promoter.

(6) The process for producing an amylopectin-type starch according to claim 1, wherein said promoter comprises a patatin I promoter.

(7) The process for producing an amylopectin-type starch according to claim 1, wherein said promoter comprises a GBSS promoter.

(8) The process for producing an amylopectin-type starch according to claim 7, wherein said GBSS promoter has the nucleotide sequence of SEQ ID No. 4.

March 1, 1999 - Visser filed "Visser Request To Add Hofvander's Patent To Interference Pursuant To 37 CFR § 1.642 (Paper No. 141).

July 18, 2001 - Final Oral Hearing.

2. Interference-in-fact

We consider first Visser's Preliminary Motion 1 (Paper No. 17) under 37 CFR § 1.633(b) for judgment that there is no interference-in-fact because none of Visser's claims designated as corresponding to the count is directed to the "same patentable invention" as any of Hofvander's claims designated

as corresponding to the count (Paper No. 17, p. 2, para. 2).[6] Visser's "motion for judgment on the ground that there is no interference-in-fact . . . is proper . . . [since] no claim of a party which corresponds to a count is identical to any claim of an opponent which corresponds to that count. See § 1.637(a)" (37 CFR § 1.633(b)).

"A party filing a motion has the burden of proof to show that it is entitled to the relief sought in the motion." 37 CFR § 1.637(a). In this case, to be entitled to the relief Visser seeks, i.e., a conclusion that there is no interference-in-fact between the inventions to which Hofvander's and Visser's claims designated as corresponding to the count Visser's and Hofvander's are directed, Visser must establish by a preponderance of the evidence of record that no claim in its involved application is directed to the same patentable invention as a claim in

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[6] We will consider the merits of Hofvander's motion to suppress (Paper No. 123) only to the extent we rely upon the evidence to which Hofvander objects (1) in concluding there exists no interference-in-fact between subject matter claimed in Visser's application and subject matter claimed in Hofvander's application and patent, or (2) in deciding unrelated preliminary motions after having concluded that there exists an interference-in-fact between subject matter claimed in Visser's involved application and subject matter claimed in Hofvander's involved application. Should we conclude that that no interference-in-fact exists between subject matter claimed in Visser's involved application and subject matter claimed in Hofvander's involved application, other preliminary or miscellaneous motions filed in this interference will be entertained only to the extent justice requires (37 CFR § 1.655 (c).

Hofvander's involved application. See 37 CFR §§ 1.601(i) and (j) below (underlining added):

> (i) An interference is a proceeding instituted in the Patent and Trademark Office before the Board to determine any question of patentability and priority of invention between two or more parties claiming the same patentable invention.
>
> (j) An interference-in-fact exists when at least one claim of a party that is designated to correspond to a count and at least one claim of an opponent that is designated to correspond to the count define the same patentable invention.

37 CFR § 1.601(n) explains the meaning of "same patentable invention" and "separate patentable invention" as follows:

> Invention "A" is the same patentable invention as an invention "B" when invention "A" is the same as (35 U.S.C. 102) or is obvious (35 U.S.C. 103) in view of invention "B" assuming invention "B" is prior art with respect to invention "A". Invention "A" is a separate patentable invention with respect to invention "B" assuming invention "A" is new (35 U.S.C. 102) and non-obvious (35 U.S.C. 103) in view of invention "B" assuming invention "B" is prior art with respect to invention "A".

Preliminarily, Visser argues both that none of its claims designated as corresponding to the count is directed to the same patentable invention as any of the claims of Hofvander's involved application which are designated as corresponding to the count (Paper No. 17, p. 2, para. 2) and that none of its claims designated as corresponding to the count is directed to the same patentable invention as any of the claims of Hofvander's U.S. Patent 5,824,798 (Paper No. 141). If we are convinced by the

evidence of record that none of Visser's claims designated as corresponding to the count is directed to the same patentable invention as any of the claims of Hofvander's involved application which are designated as corresponding to the count, we shall conclude that Visser's claims designated as corresponding to the count not only are directed to a separate patentable invention from the claims of Hofvander's involved application but prima facie are directed to a separate patentable invention from method Claims 1-8 of the Hofvander patent which Visser asks to be added to this interference pursuant to 37 CFR § 1.642 (Paper No. 141). If we are not convinced by Visser's motion, we shall independently consider Visser's § 1.642 request (Paper No. 141).

We conclude that the processes for producing an amylopectin-type starch from potato plants grown from potato tissue having a genome transformed by a PGBSS gene fragment essentially having a nucleotide sequence selected from SEQ ID No. 1, SEQ ID No. 2, and SEQ ID NO. 3 inserted in the antisense direction which are claimed in Hofvander's patent, irrespective of their having been characterized in one or more of Hofvander's applications as directed to inventions independent and distinct from the PGBSS gene fragments, antisense constructs including the PGBSS fragments, potato plant cells or tissue transformed by said antisense constructs, and potato plants grown from the

transformed plant cells or tissue which are utilized in the later patented process claims for purposes of restriction under 37 CFR § 1.142, prima facie are directed to separate patentable inventions from the subject matter claimed in Visser's involved application if we conclude that the PGBSS gene fragments, antisense constructs including said PGBSS fragments, potato plant cells or tissue transformed by the antisense constructs, and potato plants grown from the transformed plant cells or tissue required to carry out the processes claimed in Hofvander's patent are directed to separate patentable inventions from the subject matter claimed in Visser's involved application. Hofvander's reliance on an examiner's preliminary administrative determination that claims drawn to compounds are independent and distinct from claims directed to processes of using said compounds for purposes of restriction under 37 CFR § 1.142 as sole basis for a holding that the claims of Hofvander's involved application and the claims of Visser's involved application are directed to the separate patentable inventions from any of the process claims of Hofvander's patent is legally incorrect absent a comprehensive fact-specific analysis. See In re Ochiai, 71 F.3d 1565, 1572, 37 USPQ2d 1127, 1133 (Fed. Cir. 1995):

> The use of per se rules, while undoubtedly less laborious than a searching comparison of the claimed invention - including all its limitations - with the teachings of the prior art, flouts section 103 and the fundamental case law applying it. Per se rules that

> eliminate the need for fact-specific analysis of claims and prior art may be administratively convenient . . . . But, reliance on per se rules of obviousness is legally incorrect . . . . Any such administrative convenience is simply inconsistent with section 103 . . . .

We observe that Visser's § 1.642 request subsumes the main issue raised by its § 1.633(b) motion. 37 CFR § 1.642 reads:

> During the pendency of an interference, if the administrative patent judge becomes aware of an application or a patent not involved in the interference which claims the same patentable invention as a count in the interference, the administrative patent judge may add the application or patent to the interference on such terms as may be fair to all parties.

Accordingly, should we conclude that no claim of Hofvander's involved application is directed to the same patentable invention as a claim of Visser's involved application, Visser's § 1.642 request then shall be dismissed as moot.

A. Claim interpretation

(1) Claim language

We proceed to interpret the meaning of the various terms used by the parties to define the subject matter encompassed by each of the following representative claims designated as corresponding to the count so to facilitate our comparison of their respective claims:

Hofvander's Claim 4

> A fragment of a potato gene coding for . . . GBSS . . . selected from the group consisting of SEQ No. 1, SEQ ID No. 2 and SEQ ID No. 3.

Hofvander's Claim 6

An isolated potato gene coding for . . . GBSS . . . having the nucleotide sequence stated in SEQ ID No. 5.

Visser's Claim 23

A homologous construct . . . comprising a full length potato . . . GBSS cDNA or genomic DNA.

Hofvander's Claim 7

An antisense construct for inhibiting expression of the potato gene which codes for . . . GBSS . . . comprising:

> . . . a promoter, and
> . . . a fragment of a potato gene coding for . . . GBSS inserted in the antisense direction . . . selected from the group consisting of SEQ No. 1, SEQ ID No. 2 and SEQ ID No. 3.

Visser's Claim 24

The homologous construct . . . [comprising a full length potato] . . . GBSS cDNA or genomic DNA in reverse orientation.

Hofvander's Claim 13

A potato plant whose genome comprises the antisense construct . . . [for inhibiting expression of the potato gene which codes for . . . GBSS . . . comprising:

> . . . a promoter, and
> . . . a fragment of a potato gene coding for . . . GBSS inserted in the antisense direction . . . selected from the group consisting of SEQ No. 1, SEQ ID No. 2 and SEQ ID No. 3].

Visser's Claim 1

A . . . potato plant which . . . has a genome containing at least one gene construct containing a full length . . . GBSS cDNA or genomic DNA sequence coding for . . . [potato] GBSS in reverse orientation in an expression cassette . . . , said gene construct giving

> rise to tubers containing essentially amylose free starch; wherein said expression cassette comprises in the 5'-3' direction of transcription: an upstream promoter sequence, a base sequence for transcription into mRNA . . . , wherein the coding strand of said base sequence for transcription comprises an inverted sequence of bases complementary to a run of bases of . . . [potato] GBSS mRNA, wherein the transcript of said base sequence for transcription substantially inhibits the expression of . . . [potato] GBSS.

Hofvander and Visser present claims directed to methods of using antisense constructs defined by Hofvander's Claim 7 and/or Visser's Claim 15 to transgenically modify the genome of potato plant cells by genetic engineering (Hofvander's Claim 7) so to grow and regenerate potato plants with "suppress[ed] . . . amylose formation" (Hofvander Claims 7, 12-16, and 19-20), and to transgenically modify the genome of potato plant cells by genetic engineering (Visser's Claim 1) so to grow and regenerate potato plants with "tubers containing essentially amylose free starch" (Visser's Claim 1) by "substantially inhibit[ing] . . . expression of potato . . . [GBSS]" (Visser's Claim 15). Hofvander's claims designated as corresponding to the count also include methods "for tuber-specific expression of a gene product in potato, comprising transforming said potato with a DNA molecule comprising an isolated promoter from the potato gene coding for . . . GBSS . . ." (Hofvander's Claims 21 and 23) and methods "of suppressing amylose formation in potato . . . comprising a fragment of the potato gene which codes for

formation of . . . GBSS . . . inserted in the antisense direction, wherein said fragment has the nucleotide sequence of SEQ ID No. 1" (Hofvander's Claim 50).

(2) Preliminary matters

(a) Hofvander's SEQ ID Nos. 1, 2, 3, 4, and 5

Hofvander's PCT publication, published July 9, 1992 (Hofvander's PCT application, filed December 20, 1991) and Hofvander's involved application, as filed November 24, 1993 (HR 275-316), characterize SEQ ID No. 5 as follows (HR 302):

SEQ ID No. 5

Sequenced molecule: genomic DNA
Name: GBSS gene from potato
Length of sequence: 4964 bp

Hofvander's involved application, filed November 24, 1993, as amended November 17, 1993, further characterizes SEQ ID No. 5 as follows:

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4964 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

Hofvander's PCT application (p. 3, l. 21-24) and Hofvander's involved application (p. 3, l. 21-24)(HR 277) state, "The gene for potato GBSS has . . . so far not been characterised [sic] to the same extent as the waxy gene in maize, either with respect of locating or structure." Regarding the state of the art with

respect to the potato GBSS gene and its use for inhibiting amylose production in potato plant tubers, Hofvander's involved application teaches (p. 4, l. 28, to p. 5, l 22 (HR 278-279); emphasis added):

> In potato, experiments have previously been made to inhibit the synthesis of the granule-bound starch synthase (GBSS protein) with an antisense construct corresponding to the gene coding for GBSS (this gene is hereafter called the "GBSS gene"). Hergersberg (1988) [(VDX 1)] describes a method by which a cDNA clone for the GBSS gene in potato has been isolated by means of a cDNA clone for the wx$^+$ gene in maize. An antisense construct based on the entire cDNA clone was transferred to leaf discs of potato by means of Agrobacterium tumefaciens. In microtubers induced in vitro from regenerated potato sprouts, a varying and very weak reduction of the amylose content was observed and shown in a diagram. A complete characterization of the GBSS gene is not provided.
>
> The gene for the GBSS protein in potato has been further characterised [sic] in that a genomic wx$^+$ clone was examined by restriction analysis. However, the DNA sequence of the clone has not been determined (Visser et al, 1989[7]).
>
> Further experiments with an antisense construct corresponding to the GBSS gene in potato have been reported. The antisense construct which is based on a cDNA clone together with CaMV 35S promoter has been transformed by means of Agrobacterium rhizogenes. According to information, the transformation resulted in a lower amylose content in the potato, but no values have been accounted for (Flavell, 1990).

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[7] Visser, R.G.F., Hergersberger, M., van der Leij, F.R., Jacobsen, E., Witholt, B. and Feenstra, W.J. (Visser's 1989 publication), "Molecular Cloning and Partial Characterization of the Gene for Granule-Bound Starch Synthase from a Wildtype and an Amylose-Free Potato (Solanum Tuberosum L.)," Plant Science, Vol. 64, pp. 185-192 (1989)(Appendix A).

> None of the methods used so far for genetically engineered modification of potato has resulted in potato with practically no amylose-type starch.
>
> The object of the invention therefore is to provide a practically complete suppression of the formation of amylose in potato tubers.

According to Hofvander's involved application (p. 5, l. 30, to p. 6, l. 20 (HR 279-280); emphasis added):

> The antisense constructs according to the invention comprise both coding and noncoding parts of the GBSS gene which correspond to sequences in the region comprising promoter as well as leader sequence, translation start, translation end and trailer sequence in the antisense direction. For a tissue-specific expression, i.e. the amylose production should be inhibited in the potato tubers only, use is made of promoters which are specifically active in the potato tuber. As a result, the starch composition in other parts of the plant is not affected, which otherwise would give negative side-effects.
>
> The invention thus comprises a fragment which essentially has one of the nucleotide sequences stated in SEQ ID No. 1, SEQ ID No. 2 or SEQ ID No. 3. However, the sequences may deviate from those stated by one or more non-adjacent base pairs, without affecting the function of the fragments.
>
> The invention also comprises a potato-tuber-specific promoter comprising 987 bp which belongs to the gene according to the invention, which codes for granule-bound starch synthase. Neither the promoter nor the corresponding gene has previously been characterised [sic]. The promoter sequence is stated in SEQ ID No. 4, while the gene sequence is stated in SEQ ID No. 5. Also the promoter and gene sequences may deviate from those stated by one or more non-adjacent base pairs, without affecting their function.

Hofvander's involved application describes its Figure 2 (HR 312; VDX 10) as follows (HR 281, l. 1-2), "Fig. 2 shows the result of restriction analysis of the potato GBSS gene."

The specification of Hofvander's involved application explains how the Figure 2 GBSS gene was characterized (HR 282, l. 1-24):

> A full-length clone of the potato GBSS gene, wx311, has been identified and isolated from the genomic library. The start of the GBSS gene has been determined at an EcoRI fragment which is called fragment w (3.95 kb). The end of the GBSS gene has also been determined at an EcoRI fragment which is called fragment X (5.0 kb). A BgIII-SpeI fragment which is called fragment m (3.9 kb) has also been isolated and shares sequences both from fragment w and from fragment x. The fragments w, m and x have been subcloned in pUC13 (Viera, 1982; Yanisch-Peron et al, 1985) and are called pSw, pSm and pSx, respectively (Fig. 2).
>
> The GBSS gene in potato has been characterized by restriction and cDNA probes, where the 5' and 3' end of the GBSS gene has been determined more accurately (Fig. 2). Sequence determination according to Sanger et al, 1977 of the GBSS gene has been made on subclones from pSw and pSx in M13mp18 and mp19 as well as pUC19 starting around the 5' end (see SEQ ID No. 5).
>
> The promoter region has been determined as a Bg1II[sic BgIII]-NsiI fragment (see SEQ ID No. 4). Transcription and translation start has been determined at an overlapping Bg1II[sic BgIII]-HindIII fragment. The terminator region has in turn been determined at a SpeI-HindIII fragment.

The specification of Hofvander's involved application further teaches that "[t]he GBSS gene fragments according to the invention (see SEQ ID Nos 1, 2 and 3, and Fig. 2) were determined in the following manner . . ." (HR 282, l. 26-28):

> The restriction of pSw with NsiI and HindIII gives fragment I (SEQ ID No. 1) which subcloned in pUC19 is called 19NH35. Further restriction of 19NH35 with HpaI-SstI gives a fragment containing 342 bp of the gene according to the invention. This fragment comprises leader sequence, translation start and the first 125 bp of the coding region.

The restriction of pSm with HpaI and NsiI gives fragment II (SEQ ID No. 2) which subcloned in pJRD184 (Heusterpreute et al, 1987) is called pJRDmitt. Further restriction of pJRDmitt with HpaI-SstI gives a fragment containing 2549 bp of the GBSS gene according to the invention. This fragment comprises exons and introns from the middle of the gene.

The restriction of pSx with SstI and SpeI gives fragment III (SEQ ID No. 3) which subcloned in pBluescript (Melton et al, 1984) is called pBlue3'. Further restriction of pBlue3' with BamHI-SstI gives a fragment containing 492 bp of the GBSS gene according to the invention. This fragment comprises the last intron and exon, translation end and 278 bp of trailer sequence.

Hofvander's PCT publication, published July 9, 1992 (Hofvander's PCT application, filed December 20, 1991) and Hofvander's involved application, as filed November 24, 1993 (HR 275-316), characterize SEQ ID Nos. 1, 2, 3, and 4 as follows (HR 295; HR 296; HR 300; and HR 301, respectively):

SEQ ID No. 1

Sequenced molecule: genomic DNA
Name: GBSS gene fragment from potato
Length of sequence: 342 bp

SEQ ID No. 2

Sequenced molecule: genomic DNA
Name: GBSS gene fragment from potato
Length of sequence: 2549 bp

SEQ ID No. 3

Sequenced molecule: genomic DNA
Name: GBSS gene fragment from potato
Length of sequence: 492 bp

SEQ ID No. 4

Sequenced molecule: genomic DNA
Name: Promoter for the GBSS gene fragment from potato
Length of sequence: 987 bp

Hofvander's involved application, filed November 24, 1993, as amended November 17, 1993, further characterizes SEQ ID Nos. 1, 2, 3, and 4 as follows:

(2) INFORMATION FOR SEQ ID NO:1:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 342 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: DNA (genomic)

(2) INFORMATION FOR SEQ ID NO:2:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 2549 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: DNA (genomic)

(2) INFORMATION FOR SEQ ID NO:3:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 492 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: DNA (genomic)

(2) INFORMATION FOR SEQ ID NO:4:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 987 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: DNA (genomic)

(b) Visser's full length GBSS cDNA or genomic DNA

According to Visser's involved application, despite "not too encouraging" (VR 147, l. 6) results from earlier efforts to stably introduce antisense gene constructs from maize genomic GBSS into potato plants (VR 144, l. 7, to 147, l. 6), it was "nevertheless decided to expand the investigations to homologous constructs derived from a full-length potato GBSS cDNA" (VR 147, l. 6-8; emphasis added). All the genetic constructs claimed in Visser's involved application for use in creating its claimed transgenic potato plants, tubers with an "essentially amylose-free starch" (VR 140, l. 5-6) composition, and methods for creating transgenic potato plants which produce tubers with an "essentially amylose-free starch" (VR 140, l. 5-6) composition, are constructs of "full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA" (Visser's Claims 1, 15, and 23; emphasis added). Accordingly, we must first determine the meaning of the term "full length" in the phrase "full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA" from which the "construct[s] . . . [containing (Claims 1 and 15) or comprising (Claim 23)] a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA" of Visser's Claims 1, 15, and 23 are to be made before we can compare full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA to the SEQ ID Nos. 1, 2, and 3 of Hofvander's

"construct[s] . . . comprising . . . a promoter [and a] fragment selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3" (Hofvander's Claim 7); SEQ ID No. 4 of Hofvander's "DNA molecule comprising an isolated promoter from the potato gene coding for . . . GBSS . . ." (Hofvander's Claims 21 and 23), e.g., "said promoter having the nucleotide sequence . . . SEQ ID No. 4" (Hofvander's Claim 23); and SEQ ID No. 5 of Hofvander's "[i]solated potato gene coding for . . . GBSS . . . having the nucleotide sequence stated in SEQ ID No. 5" (Hofvander's Claim 6).

"To ascertain the true meaning of . . . claim language, resort should be made to the claims at issue, the specification, and the prosecution history." Minnesota Mining and Mfg. Co. v. Johnson & Johnson Orthopaedics, Inc., 976 F.2d 1559, 1576, 24 USPQ2d 1321, 1335 (Fed. Cir. 1992). "Claim interpretation involves a review of the specification, the prosecution history, the claims (including unasserted as well as asserted claims), and, if necessary, other extrinsic evidence, such as expert testimony." Hormone Research Foundation, Inc. v. Genentech, Inc., 904 F.2d 1558, 1562, 15 USPQ2d 1039, 1043 (Fed. Cir. 1990). Quoting from Johnston v. IVAC Corp., 885 F.2d 1574, 1579-80, 12 USPQ2d 1382, 1386 (Fed. Cir. 1989), the court in North Am. Vaccine, Inc. v. American Cyanamid Co., 7 F.3d 1571, 28 USPQ2d

1333 (Fed. Cir. 1993), cert. denied, 511 U.S. 1069 (1994), stated at 1575, 28 USPQ2d at 1336:

> "[C]laim interpretation may be resolved as an issue of law . . . taking into account the specification, prosecution history or other evidence." . . . .
>
> In construing claims, we begin with the language of the claims themselves. Smith-Kline Diagnostics, Inc. v. Helena Lab. Corp., 859 F.2d 878, 882, 8 USPQ2d 1468, 1472 (Fed. Cir. 1988). . . .
>
> When the meaning of a claim term is in doubt, we look to the specification for guidance. See Hormone Research Foundation, Inc. v. Genentech, Inc., 904 F.2d 1558, 1562, 15 USPQ2d 1039, 1042 (Fed. Cir. 1990) . . . .

Based on Claims 1 and 15 of Visser's involved application, we conclude that full length PGBSS cDNA or genomic DNA of Visser's Claims 1 and 15 must code for PGBSS in its functional [sense] orientation. Visser's method Claims 1 and 15 both utilize "at least one gene construct containing a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence coding for PGBSS in reverse orientation in an expression cassette which is functional in potato plants . . ." (emphasis added). These "gene construct[s] giv[e] . . . rise to tubers containing amylose free starch" (Visser's Claim 1). The "full length potato . . . GBSS . . . cDNA or genomic DNA sequence coding for PGBSS in reverse orientation" does not itself include either the "upstream promoter base sequence" (Visser's Claims 1 and 15) or "downstream transcription terminator base sequence"

(Visser's Claims 1 and 15) which is necessary for the gene construct to give rise to tubers containing amylose free starch. The "full length potato . . . GBSS . . . cDNA or genomic DNA sequence coding for PGBSS in reverse orientation" is part of a construct "in an expression cassette which is functional in potato plants" (Visser's Claims 1 and 15). The "expression cassette comprises in the 5'-3' direction of transcription" (Visser's Claim 1):

> an upstream promoter sequence, a base sequence for transcription into mRNA under control of said upstream promoter base sequence comprising coding and template strands, and a downstream transcription terminator base sequence.

We conclude from the above that both "full length potato . . . GBSS . . . cDNA or genomic DNA sequence coding for PGBSS in reverse orientation" of Visser's Claims 1 and 15 and "full length potato . . . GBSS . . . cDNA or genomic DNA . . . in reverse orientation" of Visser's Claims 23 and 24 are (Claim 1):

> . . . coding . . . base sequence[s] for transcription compris[ing] . . . an inverted sequence of bases complementary to a run of bases of PGBSS mRNA, wherein the transcript of said base sequence for transcription substantially inhibits the expression of PGBSS.

It remains unclear, however, whether fragments of a sequence of bases which do not include the complete code for PGBSS in its functional or sense orientation yet are complementary to a run of bases of PGBSS mRNA, wherein the transcript of said inverted fragment of a sequence of bases for transcription substantially

inhibits the expression of PGBSS, is a "full length potato . . . GBSS . . . cDNA or genomic DNA sequence" (Visser's Claim 23) "in reverse orientation" (Visser's Claim 24) within the meaning of the phrases in Claims 23 and 24 of Visser's involved application.

Visser's dependent Claim 14 limits the base sequence for transcription in the construct utilized in Visser's method Claim 1 to "a sequence of bases complementary to the sequence as set forth in Figure 3" (Visser's Claim 14), but it does not define "a full length potato . . . GBSS . . . cDNA or genomic DNA sequence . . . in reverse orientation" of Visser's Claim 24. Accordingly, we look to the specification of Visser's involved application for clarification.

The Summary of the Invention in Visser's involved application (VR 148, l. 1-16) refers to "at least one gene construct containing a potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA sequence in reverse or functional orientation . . . giving rise to tubers containing essentially amylose-free starch" (VR 148, l. 3-8). There, the GBSS cDNA or genomic DNA sequence is limited by its antisense function, but it is not otherwise structurally defined so to enable us to compare it to any of Hofvander's SEQ ID Nos. 1, 2, 3, 4, or 5. Visser's involved application acknowledges (VR 149, l. 6-11):

> The sense or anti-sense PGBSS cDNA or genomic DNA sequence does not have to cover the complete coding sequence but should cover a sufficient part of it to be

effective for obtaining tubers containing essentially amylose-free starch.

Visser's involved application describes the construction of the pGB50 (antisense) and pGB60 (sense) GBSS vectors depicted in its Figure 1 (VR 182) as follows (VR 149, l. 17-26):

> The original GBSS cDNA which contained an internal EcoRI site was subcloned as two fragments in pUC9, denoted pWx 1.1 and pWx 1.3. The 1.3 kb GBSS cDNA fragment from pWx 1.3 was ligated into the partial EcoRI-restricted plasmid pWx 1.1 yielding pGB2. Plasmid pGB2 was restricted with SpeI, made blunt ended with Klenow enzyme, BamHI. The GBSS cDNA fragment was ligated into BamHI-restricted pUC18 yielding pGB6 and into BamHI-digested calf intestinal phosphatase (CIP) treated pROK-1 yielding pGB50 (antisense) and pGB60 (sense).

The antisense (pGB50) and sense (pGB60) were the vectors purportedly used to transform potato plants (VR 154) and substantially inhibit the expression of PGBSS therein (VR 156-158).

We consider now the prosecution history of Visser's involved application and other evidence. Since Visser's 1989 publication (Appendix A) is cited for its background and comparable description in Visser's involved application (VR 143, 151, 152, and 170), we look first to its disclosure. Visser's 1989 publication teaches (Appendix A, p. 187, col. 1; citations omitted):

> The potato GBSS cDNA was isolated from a cDNA library established from . . . potato tubers . . . . Subcloning of the cDNA in plasmid pUC9 yielded plasmids pWx 1.1 (5'-end of the potato GBSS cDNA[)], and pWx 1.3 (3'-end of the potato cDNA) and pGB6 (pUC18 with the

two EcoRI cDNA fragments from pWx 1.1 and pWx 1.3).

. . . .

Visser's 1989 publication explains that the ligated 2.4-kb insert from pGB6 contained the total potato GBSS cDNA (Appendix A, p. 187, col. 2). Visser's 1989 publication reports that two "full length genomic clones" (Appendix A, p. 188, Figure 2, $LGBSS^{wt}$-6 and 41) were identified because they contained two EcoRI fragments and three HindIII fragments (Appendix A, p. 189, cols. 1-2, bridging para.) and hybridized to mRNA of about 2.4 kb from amylose-free tubers of mutant amf-1 (Appendix A, p. 190, col. 1). In short, Visser's 1989 publication teaches that total potato GBSS cDNA is no more than 2.4kb as indicated in VDX 2 and HDX 8.

Next, we look to the prosecution history of Visser's involved application. In various official actions mailed in Visser's involved, parent and grandparent applications, the examiner rejected one of more of Visser's claims under 35 U.S.C. § 102 and/or under 35 U.S.C. § 103 citing Hergersberg (VDX 1), Hovenkamp-Hermelink (VDX 9), Visser's PhD Thesis (VDX 7), and/or Visser's 1991 publication (VDX 8)(Paper Nos. 8, 11, 18, and 24 of Visser's grandparent, parent, and involved applications). In response thereto, applicant Visser et al. filed substantially identical Declarations under 37 CFR § 1.132 by Richard G.F. Visser (Paper No. 17 of Visser's parent application and Paper

No. 27 of Visser's involved application (Appendix B)) "to demonstrate the expression of the [GBSS] gene under the control of the promoter" (Appendix B, p. 2, first full para.). The following is said to have been performed (Appendix B, pp. 2-4):

> A series of eleven antisense constructs was made based on GBSS cDNA and genomic sequences, the 35S CaMV promoter and the GBSS promoter (Fig. 2). The construction of pGB50 has been described before (Visser et al., Mol. Gen. Genet., 225:289-26 [sic 289-296] (1991)) [(VDX 8)]. . . . .
>
> For the construction of pKGBA50 . . . the 2.2kb BamHI-SpeI fragment from pGB2 . . . was ligated in reversed orientation into digested pPGB-1S. For the construction of pGBA10 and pKGBA10 the 3.0kb HindIII-SpeI fragment containing the complete coding region of the GBSS gene . . . was subcloned in pUC19 (=SUB10; Fig. 2a). The BamHI-SpeI fragment of SUB10 was ligated in reverse orientation into digested pBl121S or pPGB-1S, respectively.
>
> The partial genomic antisense constructs pGBA20, pKGBA20, pGBA30 and pKGBA30 are based on BamHI and SstI digested pBl121 and pPGB-1. The 1.8kb HindIII-Nsi1 fragment of the GBSS gene was subcloned in pMTL23 . . . and isolated as an SstI-BamHI fragment (=SUB20; Fig. 2a). This fragment was ligated in reversed orientation into pBl121 (=pGBA20) and pPGB-1 (=pKGBA20). The 1.4kb SstI-KpnI fragment of the GBSS gene was subcloned in pUC19 and isolated as an SstI-BamHI fragment (=SUB30; Fig. 2a), which was ligated in reversed orientation into pBl121 (=pGBA30) and pPGB-1 (=pKGBA30). For construction of pKGBA25 . . . PCR products were . . . restricted with SstI and XbaI and ligated in reversed orientation into XbaI-SstI digested pPGB-1. For the construction of pKGBA31 the 0.6kb SstI-SpeI fragment of the GBSS gene (=SUB31; Fig. 1a [sic, 2a]) was directly ligated in reversed orientation into XbaI-SstI digested pPGB-1.

Accompanying Visser's Declarations under 37 CFR § 1.132 is Figure 2 (Appendix B, last page). Figure 2A is said to depict:

LGBSSwt-6: the "full length genomic clone" described in Visser's 1989 publication (Appendix A, p. 188) ("The line on top indicates the gene including the promoter region (5'dashed line) and the terminator region (3' dashed line).) (Appendix B);

SUB10: the 3.0kb HindIII-SpeI fragment of the GBSS gene containing the complete coding region of the GBSS gene subcloned in pUC19 (Appendix B, p. 3);

SUB20: the 1.8kb HindIII-Nsi1 fragment of the GBSS gene subcloned in pMTL23 . . . and isolated as an SstI-BamHI fragment (Appendix B, p. 3);

SUB25: a 1.1kb fragment of the GBSS gene amplified via PCR with a 23-mer Sst-primer at the 5' end of the fragment and a 23-mer Xba-primer at the 3' end of the fragment (Appendix B, pp. 3-4);

SUB30: the 1.4kb SstI-KpnI fragment of the GBSS gene subcloned in pUC19 and isolated as an SstI-BamHI fragment (Appendix B, p. 3);

SUB31: the 0.6kb SstI-SpeI fragment of the GBSS gene.

Figure 2B depicts, inter alia, the following constructs comprising GBSS cDNA and SUB10, SUB20, SUB25, SUB30, and SUB31 genomic DNA fragments in reverse orientation with a GBSS (GB) or 35 CaMV (35S) promoter:

35 CaMV (35S) promoter		GBSS (GB) promoter	
pGB50	35S-GBSS cDNA;	pKGBA50	GB-GBSS cDNA;
pGBA10	35S-SUB10;	pKGBA10	GB-SUB10;
pGBA20	35S-SUB20;	pKGBA20	GB-SUB20;
		pKGBA25	GB-SUB25;
pGBA30	35S-SUB30;	pKGBA30	GB-SUB30;
		pKGBA31	GB-SUB31.

While Visser's Rule 132 declaration was expressly designed to demonstrate unexpectedly superior expression of GBSS cDNA and

GBSS cDNA and genomic DNA fragments under the control of the GBSS promoter as compared to control by the 35 CaMV promoter, it also defines the antisense pGB50 vectors said in Visser's involved application to have been used to transform potato plants (VR 154) and substantially inhibit the expression of PGBSS (VR 156-158) as constructs comprising GBSS cDNA which corresponds to genomic SUB10. SUB10 is defined as a 3.0kb HindIII-SpeI fragment of the GBSS gene containing the complete coding region of the GBSS gene in reverse orientation and an upstream 35S CaMV promoter (Appendix B, p. 3).

The above additional information is particularly significant because it lays a firm basis for interpreting the metes and bounds of the phrase "full length potato . . . GBSS . . . cDNA or genomic DNA sequence . . . in reverse orientation" in all claims of Visser's involved application. Figures 2A and 2B of Visser's Rule 132 declaration (Appendix B, last page) depict what reasonably appears to be the same LGBSSwt-6 clone, the same gene including the 5' promoter and the 3' terminator regions, and the same GBSS gene fragments SUB10, SUB20, SUB25, SUB30, and SUB31 which form the same pGB50, pKGBA50, pGBA10, pKGBA10, pGBA20, pKGBA20, pKGBA25, pGBA30, pKGBA30, pKGBA31 gene constructs depicted in Figures 1A and 1B (VDX 4, p. 748) of Kuipers et al.

(Kuipers' 1995 publication)[8], "Factors Affecting the Inhibition by Antisense RNA of Granule-Bound Starch Synthase Gene Expression in Potato," Mol. Gen. Genet., Vol. 246, pp. 745-755 (1995) (VDX 4). Figure 1B of Kuipers' 1995 publication additionally depicts a pKGBA55 construct which is said to include the GBSS cDNA fragment corresponding to SUB25 (VDX 4, p. 749, Fig. 2A-C). Most significant is the additional description in Kuipers' 1995 publication of the GBSS cDNA, genomic DNA, and genomic GBSS DNA fragments which were used to form the pGB50, pKGBA50, pGBA10, pKGBA10, pGBA20, pKGBA20, pKGBA25, pGBA30, pKGBA30, pKGBA31 gene constructs depicted in its Figures 1A and 1B (VDX 4, p. 748), and as the evidence as a whole reasonably indicates, were also used to form the pGB50, pKGBA50, pGBA10, pKGBA10, pGBA20, pKGBA20, pKGBA25, pGBA30, pKGBA30, pKGBA31 gene constructs depicted in corresponding Figures 2A and 2B of Visser's Rule 132 declaration (Appendix B, last page).

Kuipers' 1995 publication states (VDX 4, p. 749, Fig. 2A-C) (emphasis added):

> Fig. 2A-C Evaluation of the influence of antisense construct composition on the degree of inhibition of GBSS gene expression. A <u>Full-length genomic DNA (pGBA10 and pKGBA10) versus full-length GBSS cDNA (pGB50 and pKGBA50)</u>. B Internal fragment genomic GBSS DNA (pKGBA25) versus corresponding fragment of GBSS cDNA (pKGBA55). C 35S CaMV promoter (pGBA10

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[8] Named authors are Anja G.J. Kuipers, Wim J.J. Soppe, Evert Jacobsen, and Richard G.F. Visser (VDX 4, p. 745).

> and pGB50) versus GBSS promoter (pKGBA10 and pKGBA50). Within parentheses are the numbers of individual transformants. (complete complete inhibition of GBSS gene expression, incomplete incomplete inhibition of GBSS gene expression, no inhibition no inhibition of GBSS gene expression)[.]

In its discussion, "Effect of construct composition on antisense inhibition: cDNA versus genomic DNA" (VDX 4, p. 752, col. 1), Kuipers' 1995 publication discloses (emphasis added):

> The origin of the GBSS sequence was shown to be an important factor in determining the efficacy of antisense inhibition. The full-length GBSS cDNA (pGB50, pKGBA50) and genomic DNA (pGBA10, pKGBA10) constructs were all found to be capable of complete inhibition of GBSS gene expression, but it was shown that the antisense GBSS cDNA constructs resulted in complete inhibition of GBSS gene expression in a higher percentage of transgenic potato clones (Table 1). This was also observed for the partial cDNA construct pKGBA55 as compared to the corresponding partial genomic construct pKGB25. The percentage of clones with inhibited GBSS gene expression was shown to be higher for the antisense GBSS cDNA constructs than for the genomic DNA constructs (Fig. 2A). The presence of intron sequences in the genomic constructs might contribute to the observed differences in antisense inhibition. The full length GBSS gene contains 12 introns (van der Leij et al. 1991), four of which are also present in the gene fragment used for pKGBA25. These introns will not be processed when present in antisense orientation. . . . The supposed . . . can be explained by the differences in the GC content, which is 42.7% for exon (cDNA) sequences and 33% for intron sequences. . . . In this way, the presence of intron sequences with a low GC content might reduce the efficacy of antisense inhibition of gene expression.

In its discussion, "Effect of construct composition on antisense inhibition: full-length versus partial genomic DNA" (VDX 4, p. 752, col. 2; emphasis added), Kuipers' 1995 publication discloses:

> In transgenic clones, the degree of inhibition of GBSS gene expression was found to vary for the genomic GBSS antisense constructs. However, similar frequencies of complete and incomplete inhibition could be achieved with pGBA10, pKGBA10 and pKGBA31 (comprising 0.6kb of the 3' end of the GBSS coding region and containing one intron sequence). This indicates that the size of the antisense RNA does not affect the efficacy of inhibition. Furthermore, it demonstrates that the GBSS fragment used in pKGBA31, or at least part of it, is essential for the inhibition of GBSS gene expression, as the inhibitory effect of pGBA20, pKGBA20 and pKGBA25 was much lower.
>
> For pGBA30 and pKGBA30, the weak inhibitory effect may be caused by a premature transcription termination. The genomic fragment used for these constructs contains a 3' non-GBSS sequence, which comprises a part of a putative pseudogene (van der Leij et al. 1993), in addition to the GBSS fragment that is also present in pKGBA31. . . . A premature transcription stop does not necessarily result in the absence of antisense inhibition, as has been described for pGB50 (Kuipers et al. 1994) and several other antisense genes . . . but in the case of pGBA30 and pKGBA30 the resulting antisense RNA might lack sequences that are complementary to the GBSS mRNA.
>
> The variation in the inhibitory effects of the partial genomic antisense constructs points towards a function for certain regions of the gene in antisense inhibition. . . . .

(c) Construct[s] "comprising" a fragment; construct[s] "containing" a sequence; and sequence[s] "comprise[s]" sequence[s]

The following phrases appear in the claims of Hofvander's involved application (emphasis added):

Hofvander's Claim 1

". . . a gene construct comprising a fragment of the potato gene which codes for formation of granule-bound starch synthase (GBSS gene) inserted in the antisense direction, wherein said

fragment is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, together with a promoter selected from the group consisting of CAMV 35S, patatin I and the GBSS promoter";

Hofvander's Claim 7

"An antisense construct . . . comprising . . . a promoter [and] . . . a fragment of the potato gene coding for granule-bound starch synthase inserted in the antisense direction, wherein said fragment is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3";

Hofvander's Claim 10

"A vector comprising a fragment of the potato gene coding for granule-bound starch synthase (GBSS), wherein said fragment is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, and said fragment is inserted in the antisense direction";

Hofvander's Claims 21 and 23

"transforming . . . potato with a DNA molecule comprising an isolated promoter from the potato gene coding for granule-bound starch synthase"; and

Hofvander's Claim 50

"a gene construct comprising a fragment of the potato gene which codes for formation of granule-bound starch synthase (GBSS gene) inserted in the antisense direction".

The following phrases appear in the claims of Visser's involved application (emphasis added):

Visser's Claims 1 and 15

"gene construct containing . . . a full length potato . . . GBSS . . . cDNA or genomic DNA sequence coding for PGBSS in reverse orientation";

"a base sequence . . . comprising coding . . . strands"; and

"coding strand of said base sequence . . . comprises . . . an inverted sequence of bases complementary to . . . PGBSS mRNA";

Visser's Claim 13

"construct contains full length PGBSS cDNA";

Visser's Claim 14

"sequence . . . comprises . . . a sequence of bases";

Visser's Claim 16

"said construct further comprises T-DNA"; and

Visser's Claim 23

"construct . . . comprising a full length potato . . . GBSS . . . cDNA or genomic DNA".

As a matter of law, we ask first whether a gene construct or vector "comprising" a PGBSS gene fragment selected from the group consisting of SEQ ID Nos. 1, 2, or 3, or an upstream promoter and a PGBSS gene fragment selected from the group consisting of SEQ ID Nos. 1, 2, or 3 of Hofvander's claims, or a gene construct "containing" a full length PGBSS cDNA or genomic

DNA sequence coding for PGBSS in reverse orientation of Visser's claims, reads on a fragment of the PGBSS gene which includes as a subfragment thereof, a sequence identified as SEQ ID No. 1, 2, or 3, or an upstream promoter sequence and a sequence identified as SEQ ID No. 1, 2, or 3 in the case of Hofvander's claims, or a larger fragment of the PGBSS gene, including as a subfragment thereof, a sequence identified as a full length PGBSS cDNA or genomic DNA sequence in the case of Visser's claims. We must give the language of the parties' claims its broadest reasonable interpretation consistent with the supporting disclosures.

According to the respective specifications, a gene construct or vector "comprising" a PGBSS gene fragment selected from the group consisting of SEQ ID No. 1, 2, or 3, or an upstream promoter sequence and a sequence identified as SEQ ID No. 1, 2, or 3 of Hofvander's claims, or a gene construct "containing" a full length PGBSS cDNA or genomic DNA sequence coding for PGBSS in reverse orientation of Visser's claims, does not generally read on larger fragments of the PGBSS gene, each of which includes as a subfragment thereof, a sequence identified as SEQ ID No. 1, 2, or 3 or an upstream promoter sequence and a sequence identified as SEQ ID No. 1, 2, or 3 in the case of Hofvander's claims, or generally read on larger fragments of the PGBSS gene which includes as a subfragment thereof, a sequence identified as a full length PGBSS cDNA or genomic DNA sequence in the case of

Visser's claims. Hofvander's specification would have led persons having ordinary skill in the art to understand that other sequences which are PGBSS fragments substantially larger in size than the sequences identified as SEQ ID Nos. 1, 2 and 3 in reverse orientation, or gene sequences including an upstream promoter sequence and a fragment substantially larger in size than SEQ ID Nos. 1, 2 and 3 in reverse orientation are, in general, not useful to substantially inhibit expression of PGBSS in potato plants, i.e., to transform potato plants to suppress amylose formation, and not part of Hofvander's invention (HR 278-279; emphasis added):

> In potato, experiments have previously been made to inhibit the synthesis of the granule-bound starch synthase (GBSS protein) with an antisense construct corresponding to the gene coding for GBSS (this gene is hereafter called the "GBSS gene"). Hergersberg (1988) [(VDX1)] describes a method by which a cDNA clone for the GBSS gene in potato has been isolated by means of a cDNA clone for the wx^+ gene in maize. An antisense construct based on the entire cDNA clone was transferred to leaf discs of potato by means of Agrobacterium tumefaciens. In microtubers induced in vitro from regenerated potato sprouts, a varying and very weak reduction of the amylose content was observed and shown in a diagram. A complete characterization of the GBSS gene is not provided.
>
> The gene for the GBSS protein in potato has been further characterised [sic] in that a genomic wx^+ clone was examined by restriction analysis. However, the DNA sequence of the clone has not been determined (Visser et al, 1989).
>
> Further experiments with an antisense construct corresponding to the GBSS gene in potato have been reported. The antisense construct which is based on a cDNA clone together with CaMV 35S promoter has

been transformed by means of Agrobacterium rhizogenes. According to information, the transformation resulted in a lower amylose content in the potato, but no values have been accounted for (Flavell, 1990).

None of the methods used so far for genetically engineered modification of potato has resulted in potato with practically no amylose-type starch.

The object of the invention therefore is to provide a practically complete suppression of the formation of amylose in potato tubers.

Hofvander's involved application expressly states (HR 279-280; emphasis added):

The antisense constructs according to the invention comprise both coding and noncoding parts of the GBSS gene which correspond to sequences in the region comprising promoter as well as leader sequence, translation start, translation end and trailer sequence in the antisense direction. For a tissue-specific expression, i.e. the amylose production should be inhibited in the potato tubers only, use is made of promoters which are specifically active in the potato tuber. As a result, the starch composition in other parts of the plant is not affected, which otherwise would give negative side-effects.

The invention thus comprises a fragment which essentially has one of the nucleotide sequences stated in SEQ ID No. 1, SEQ ID No. 2 or SEQ ID No. 3. However, the sequences may deviate from those stated by one or more non-adjacent base pairs, without affecting the function of the fragments.

The invention also comprises a potato-tuber-specific promoter comprising 987 bp which belongs to the gene according to the invention, which codes for granule-bound starch synthase. Neither the promoter nor the corresponding gene has previously been characterised [sic]. The promoter sequence is stated in SEQ ID No. 4, while the gene sequence is stated in SEQ ID No. 5. Also the promoter and gene sequences may deviate from those stated by one or more non-adjacent base pairs, without affecting their function.

Similarly, as discussed previously, the claims, specification, and prosecution history in Visser's involved application, and other extraneous evidence of record, establish that "full length" PGBSS cDNA or genomic DNA coding for PGBSS in reverse orientation of Visser's claims means the cDNA or genomic DNA 5'-3' GBSS coding region of the PGBSS gene, not the full gene including, for example its promoter and termination sequences. Nor does the "full length" PGBSS cDNA or genomic DNA coding for PGBSS in reverse orientation of Visser's claims read on any cDNA or genomic DNA GBSS gene fragments which generally include the 5'-3' GBSS coding region of the PGBSS gene which are "sufficient . . . to be effective for obtaining tubers containing essentially amylose-free starch" (VR149, l. 8-9). While the specification of Visser's involved application suggests that "[t]he sense or anti-sense PGBSS cDNA or genomic DNA sequence does not have to cover the complete coding sequence" (VR 149, l. 6-7), Visser's claims are directed to no more nor less than the full length coding region for the GBSS gene or its corresponding cDNA and the Visser's specification would have led persons skilled in the art to use no more nor less than "full length" PGBSS cDNA or genomic DNA coding for PGBSS in reverse orientation and reasonably expect to obtain tubers containing essentially amylose-free starch without undue experimentation. Visser's specification no more than invites persons skilled in the art to experiment to further

determine what other fragments of the GBSS gene or complete coding region of the GBSS gene might also be used to genetically engineer potatoes to produce tubers containing essentially amylose-free starch.

(d) "Suppressing" amylose formation; "inhibiting expression" of PGBSS gene; "giving rise to tubers containing essentially amylose free starch"; and "substantially inhibits" expression of PGBSS gene

Claims 1 and 50 of Hofvander's involved application, and claims dependent thereon, are directed to "method[s] of suppressing amylose formation in potato . . . ." Claims 7-20 and 22 of Hofvander's involved application are directed to "antisense constructs for suppressing amylose formation in potato . . ." (Hofvander's Claims 7-9), vectors comprising the antisense constructs of Hofvander's Claim 7, and cells, potato plants, potato tubers, seeds, and microtubers whose genome comprises the antisense constructs of Hofvander's Claims 7-8. As we read the method and antisense construct claims of Hofvander's involved application consistent with the supporting specification, amylose formation in potato is suppressed by cultivating potato plants from potato plant cells transformed by the specifically identified antisense constructs also claimed for use in inhibiting expression of the PGBSS gene. Hofvander teaches and claims that amylose formation in potato plants is suppressed when one of the antisense constructs

identified in Hofvander's claims is inserted in the antisense direction into the genome of a potato cell and a potato plant is cultivated from the genetically engineered cell. In other words, the methods of suppressing amylose formation in potato to which Hofvander claims are directed require that specifically identified antisense constructs be inserted into potato plant cells to transform the potato cells and inhibit expression of the potato gene therein and in potato plants cultivated therefrom. The antisense constructs identified as being useful for transforming potato plant cells and capable of suppressing amylose formation in potato plants grown therefrom include DNA sequences specifically identified in the respective claims.

If the chemical structures of the DNA sequences in the antisense constructs Visser uses to genetically engineer potato plants by established procedures to produce essentially amylose free starch are the same, or substantially the same, as the chemical structures of the DNA sequences of the antisense constructs Hofvander describes for use in genetically engineering its potato plants also by established procedures to suppress amylose formation in potato tubers, absent any references to patentably distinguishable genetic engineering methods in the claims themselves, the different terminology the parties' respective claims employ to define function and/or specify utility does not make the same, or substantially the same,

methods and constructs both parties appear to claim separately patentable to each of them. See In re Pearson, 494 F.2d 1399, 1403, 181 USPQ 641, 644 (CCPA 1974)("[T]erms [which] merely set forth the intended use for, or a property inherent in, an otherwise old composition . . . do not differentiate the claimed composition from those known to the prior art"). In re Swinehart, 439 F.2d 210, 169 USPQ 226 (CCPA 1971), instructs at 212-213, 169 USPQ at 229:

> [I]t is elementary that the mere recitation of a newly discovered function or property, inherently possessed by things in the prior art, does not cause a claim drawn to those things to distinguish over the prior art.

See also In re Woodruff, 919 F.2d 1575, 1578, 16 USPQ2d 1934, 1936 (Fed. Cir. 1990):

> It is a general rule that merely discovering and claiming a new benefit of an old process cannot render the process again patentable. . . . . While the processes encompassed by the claims are not entirely old, the rule is applicable here to the extent that the claims and prior art overlap.

Similarly, if the DNA sequences of the antisense constructs Visser uses to genetically engineer potato plants by unspecified established procedures to produce essentially amylose free starch structurally are not the same, or substantially the same, as the DNA sequences of the antisense constructs Hofvander describes for use in genetically engineering potato plants by the same, or substantially the same, unspecified established procedures to suppress amylose formation in potato tubers, the common

terminology the parties' respective claims employ to define their function and/or utility does not establish that the subject matter one claims is patentably indistinct from the subject matter the other claims. See In re Dillon, 919 F.2d 688, 695, 16 USPQ2d 1897, 1903 (Fed. Cir. 1990)(en banc), cert. denied, 500 U.S. 904 (1991):

> Suffice it to say that we do not regard [In re] Durden[, 763 F.2d 1406, 226 USPQ 359 (Fed. Cir. 1985),] as authority to reject every method claim reading on an old type of process, such as mixing, reacting, reducing, etc. The materials used in a claimed process as well as the result obtained therefrom must be considered along with the specific nature of the process, and the fact that new or old, obvious or nonobvious, materials are used or result from the process are only factors to be considered, rather than conclusive indicators of the obviousness or nonobviousness of a claimed process. When any applicant properly presents and argues suitable method claims, they should be examined in light of all these relevant factors, free from any presumed controlling effect of Durden. Durden did not hold that all methods involving old process steps are obvious; the court in that case . . . refused to adopt an unvarying rule that the fact nonobvious starting materials and nonobvious products are involved ipso facto makes the process nonobvious. Such an invariant rule always leading to the opposite conclusion is also not the law.

After quoting from In re Dillon, supra, the court in In re Ochiai, 71 F.3d 1565, 37 USPQ2d 1127 (Fed. Cir. 1995), stated at ____, 37 USPQ2d at 1133 (emphasis added):

> Having compared Ochiai's claims, limited as they are to the use of a particular nonobvious starting material for making a particular nonobvious end product, to the prior art of record, we reverse . . . .

Having considered all the evidence in this case pertinent to interpretation of the parties' claims, we find that the similarities and dissimilarities of the functional terminology in the claims of Hofvander's and Visser's involved applications are far less significant for comparing the subject matter of the respective parties' claims than the similarities and differences in the structures of the DNA sequences in the antisense constructs used to inhibit expression of the potato gene and suppress amylose formation in potato plants. In this case, functional language and/or specified utility is insignificant because we find that the function and/or specified utility of the claimed subject matter depends on the chemical structures of the DNA sequences in the antisense constructs of the claims of the respective parties and the chemical structures of the DNA sequences in the antisense constructs of the claims of the respective parties are well defined for comparison. Functional language and/or relative degrees of utility are more significant where the chemical structures upon which all the claimed subject matter of the respective parties is based cannot be compared. Compare In re Thorpe, 777 F.2d 695, 698, 227 USPQ 964, 966 (Fed. Cir. 1985):

> Thorpe argues that even if the performance of a compound is comparable to that of the prior art, this fact does not necessarily imply that the structures are identical. We agree.

Evidence that prior art potatoes do not necessarily or inherently possess the same properties or produce the same results may become significant if the claimed and prior art antisense constructs used to transform the potato plants reasonably appear to be identical, or substantially identical, and accordingly, the potato plants reasonably appear to be transformed by identical or substantially identical processes. Compare In re Best, 562 F.2d 1252, 1255, 195 USPQ 430, 433 (CCPA 1977):

> Where . . . the claimed and prior art products are identical or substantially identical, or are produced by identical or substantially identical processes, the PTO can require an applicant to prove that the prior art products do not necessarily or inherently possess the characteristics of his claimed product.

In this case, the chemical structures of the DNA sequences of the antisense constructs the parties' claims describe are well defined and readily compared. Therefore, it unnecessary to determine the relative degrees of function indicated in the parties' claims until we find, based on comparable structures of DNA sequences, that the antisense constructs the parties respectively claim reasonably appear to be the same, or substantially the same, or conclude that the antisense constructs of one party's claims reasonably would appear to have been suggested by the other party's claims. See In re Mills, 916 F.2d 680, 683, 16 USPQ2d 1430, 1433 (Fed. Cir. 1990) ("It is not

pertinent whether the prior art . . . possesses the functional characteristics of the claimed invention if the reference does not describe or suggest its structure").

(e) "Sense" claims

Claims 4, 6, 21 and 23 of Hofvander's involved application[9] and Claim 23 of Visser's involved application[10] stand designated as corresponding to the count. Visser's Preliminary Motion 2 (VPM 2)(Paper No. 18) for judgment that Claim 6 of Hofvander's involved application, filed November 24, 1993, is unpatentable

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[9] 4. A fragment of a potato gene coding for granule-bound starch synthase (GBSS), wherein said fragment is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3.

6. Isolated potato gene coding for granule-bound starch synthase in potato (GBSS gene) having the nucleotide sequence stated in SEQ ID No. 5.

21. A method for tuber-specific expression of a gene product in potato, comprising transforming said potato with a DNA molecule comprising an isolated promoter from the potato gene coding for granule-bound starch synthase (GBSS).

23. A method for tuber-specific expression of a gene product in potato, comprising transforming said potato with a DNA molecule comprising an isolated promotor [sic] from the potato gene coding for granule-bound starch synthase (GBSS), said promoter having the nucleotide sequence stated in SEQ ID No. 4.

[10] 23. A homologous construct of the potato plant comprising a full length potato granule-bound starch synthase (PGBSS) cDNA or genomic DNA.

under 35 U.S.C. § 102 over Hergersberg (VDX 1)) or Hovenkamp-Hermelink (VDX 9); and/or under 35 U.S.C. § 103 in view of the combined teachings of Hergersberg, Hovenkamp-Hermelink, Visser's PhD Thesis (VDX 7), and van der Leij (VDX 3); was dismissed as moot with respect to Claim 6 (Paper No. 74, p. 5 n. 1):

> Since Hofvander et al. have attempted to cancel claim 6, judgment will be entered against claim 6 when final judgment is entered in this case.

At Final Hearing on July 18, 2001, Hofvander's counsel, Mr. R. Danny Huntington, stated that Claim 6 of Hofvander's involved application no longer was part of this interference.

Hofvander's Preliminary Motion 1 (HPM 1)(Paper No. 28) to substitute Proposed Count H-1 was granted (Paper No. 74, p. 10). In the course of its decision granting HPM 1, the decision on motions noted (Paper No. 74, p. 11 n. 3):

> Visser's opposition to Hofvander's motion (1) acknowledges in footnote 13 that Visser's claim 23 is unpatentable over prior art. Judgment with respect to this claim is deferred to final hearing.

Footnote 13 of Visser's opposition to Hofvander's Preliminary Motion 1 (Paper No. 28) reads (Paper No. 38, p. 11 n. 13):

> Visser agrees with Hofvander that Count 1 and corresponding Visser claim 23 are unpatentable. Visser would have cancelled claim 23 but for the fact the rules do not permit the cancellation of such a claim. Visser claim 23 (and the Count) are unpatentable to Hofvander and Visser at least in view of the teachings of Hergersberg because Visser claim 23 (and the Count) include the full length potato GBSS gene, i.e., in the sense orientation.

Visser's brief clarifies its position with respect of Claim 23 (VB 32):

> Visser claim 23 is directed to an homologous construct of the potato plant comprising a full length GBSS cDNA or gDNA. There is no recitation that the full length GBSS cDNA or gDNA is in the reverse or antisense orientation. As such, claim 23 is not patentable because it is anticipated by the expression of the GBSS gene as it naturally occurs in the potato plant. The judgment should be entered that Visser claim 23 is unpatentable.

Whether or not Hofvander's Claim 6 and Visser's Claim 23 are patentable to the respective parties, the claims are involved in this interference. "Any claim of an application or patent that is designated to correspond to a count is a claim involved in the interference within the meaning of 35 U.S.C. 135(a)." 37 CFR § 1.601(f). However, "[a]n interference is a proceeding . . . to determine any question of patentability and priority of invention between two or more parties claiming the same patentable invention" (37 CFR § 1.601(i); emphasis added). To determine whether or not an interference-in-fact exists between subject matter claimed in Hofvander's involved application and subject matter claimed in Visser's involved application in this case, we must consider whether subject matter defined by Claim 6 of Hofvander's involved application (subject matter which Hofvander no longer considers to be its invention and/or subject matter which Hofvander concedes is not independently patentable to it) is patentable to Hofvander over subject matter defined by

Claim 23 of Visser's involved application (subject matter which Visser no longer claims to be its invention and/or subject matter which Visser concedes is not independently patentable to it) and vice versa. We decline to determine patentability or priority of invention between two parties where one party concedes that the invention being claimed is unpatentable to it and the other has attempted to cancel its claim drawn to the same invention. We shall not base our determination whether or not an interference-in-fact exists in this case on the patentability of Hofvander's Claim 6 to Hofvander over prior art including Visser's Claim 23, or the patentability of Visser's Claim 23 to Visser over prior art including Hofvander's Claim 6. Since the subject matter of Claim 6 of Hofvander's involved application does not appear to be patentable to Hofvander and the subject matter of Claim 23 of Visser's involved application does not appear to be patentable to Visser, we will not determine that an interference-in-fact exists based on those claims. Interference proceedings are not designed to determine questions of patentability between two parties claiming subject matter unpatentable to one or the other. See 37 CFR § 1.601(i).

Aside from Visser's Claim 23, none of Visser's claims designated as corresponding to the count is directed to, or utilizes, a DNA construct in its sense orientation. Hofvander's Claims 4, 21 and 23, although designated as corresponding to the

count, are directed to "[a] fragment of a potato gene coding for . . . GBSS . . . selected from the group consisting of SEQ ID Nos. 1, SEQ ID No. 2 and SEQ ID No. 3" (Hofvander's Claim 4) and "method[s] for tuber-specific expression of a gene product in potato, comprising transforming said potato with a DNA molecule comprising an isolated promoter from the potato gene coding for . . . GBSS . . ." (Hofvander's Claims 21 and 23; emphasis added). Unlike Claims 1, 4-8, 11, 13-20, 22, and 24-27 of Visser's involved application, all of which require that the DNA sequences be in the antisense orientation, the inventions of Claims 4, 6, 21 and 23 of Hofvander's involved application do not appear to be directed to, or utilize, DNA sequences in antisense orientation. Visser argues (VB 38, first full para.):

> The APJ has stated that "It is Visser's position that the constructs of Hofvander are not antisense" (Paper No. 74, pp. 12-13). This is, however, only Visser's position with respect to Hofvander claims 4, 6, 21 and 23. It is Visser's position that Visser's separately patentable claims (which require the DNA sequences to be in the antisense orientation) are distinguishable over Visser claim 23 and Hofvander claims 4, 6, 21 and 23 because these claims fail to recite that the DNA sequences are in the antisense orientation.

Rather than dispute Visser's position, Hofvander replied (HB, pp. 60-61):

> While Visser calls attention to the fact that "Hofvander claims 4, 6, 21 and 23 are not directed to antisense constructs," Visser ignores the fact that the other Hofvander claims designated as corresponding to the count, claims 1, 7-20, 22 and 50, all recite

> antisense constructs. . . . Assuming arguendo, that the antisense constructs are directed to a separately patentable invention from the sense constructs, it is irrelevant to Visser. Visser has no claims in this interference directed to sense constructs. As stated in Visser's Brief, a restriction requirement was issued between antisense constructs and sense constructs. Based upon Visser's belief that Hofvander claims 4, 6, 21 and 23 relating to sense constructs are directed to a separately patentable invention, assuming that Visser filed a divisional application directed to claim 3, perhaps Visser should have filed a motion under 37 C.F.R. § 1.633(e)(1) for an additional interference between Hofvander claims 4, 6, 21 and 23 and Visser claim 3.
>
> In the instant interference, however, because Hofvander has claims directed to antisense constructs, which have been designated as corresponding to the Count, the Hofvander claims define the same patentable invention as the Visser claims. Because at least one of Hofvander's claims is directed to antisense constructs, the Visser invention directed to antisense constructs defines the same patentable invention as the Hofvander invention in accordance with 37 C.F.R. § 1.601(n). As such, this argument by Visser is once again unpersuasive.

Since the parties maintain at final hearing only that Claims 1, 7-20, 22 and 50 of Hofvander's involved application are drawn to the "same patentable invention" as Claims 1, 4-8, 11, 13-20, 22, and 24-27 of Visser's involved application, we limit our claim interpretation to, and proceed to decide the issues presented in this case with regard to, Claims 1, 7-20, 22 and 50 of Hofvander's involved application and Claims 1, 4-8, 11, 13-20, 22, and 24-27 of Visser's involved application.

(3) Findings and conclusions

Based on the claims, the supporting specification, and prosecution history of Hofvander's and Visser's involved

applications, and other extrinsic evidence of record, we find and/or conclude that:

I. Hofvander's claims are generally directed to:

(i) a construct including an a DNA fragment in the antisense direction which suppresses expression of the GBSS gene of a potato plant when it is inserted into the potato plant genome, and

(ii) a method of suppressing amylose formation in potato tubers, which comprises cultivating a potato plant which has been genetically engineered to suppress expression of the GBSS gene by insertion of a construct including a DNA fragment in the antisense direction which suppresses expression of the GBSS gene of a potato plant into the potato plant genome.

II. The construct described in Hofvander's claims for insertion and/or inserted into the potato plant genome in antisense direction includes one of three genomic DNA fragments of the 0-4964 bp PGBSS gene identified as PCT SEQ ID No. 5 (VDX 15/HDX 8).

III. The 0-4964 bp PGBSS gene may be divided into five segments of ~1000 bp designated as follows (VDX 15/HDX 8):

(i) 0-1000 bp segment,
(ii) 1000-2000 bp segment,
(iii) 2000-3000 bp segment,
(iv) 3000-4000 bp segment, and
(v) 4000-4964 bp segment.

IV. The 0-4964 bp PGBSS gene includes (VDX 15/HDX 8):

(i) a GBSS promoter region including the entire 0-1000 bp segment and a minor fraction of the 1000-2000 bp segment (~1200 bp),

(ii) a 5'-3' PGBSS coding region including a major fraction of the 1000-2000 bp segment, the entire 2000-3000 bp segment, the entire 3000-4000 bp segment, and a minor fraction of the 4000-4964 bp segment (~3000 bp); and

(iii) a terminator region including a major fraction of the 4000-4964 bp segment (~750 bp).

V. Leij (VDX 3) describes the "complete genomic nucleotide sequence" of the PGBSS gene, excluding the putative promoter sequences and polyadenylation signals (VDX 3, Fig. 1, p. 243), as having 2961 bp (~3000 bp)(VDX 3, Fig. 1, p. 243). Leij's 2961 bp 5'-3' genomic DNA sequence is the 5'-3' gDNA sequence indicated by the bold arrow above the 0-4964 bp PCT SEQ ID No. 5 in VDX 15/HDX 8.

VI. The 5'-3' genomic DNA sequence depicted by the antisense arrow at the top of Kuipers' Fig. 1A (VDX 4, p. 748/ HR 339) is defined as follows (VDX 4, p. 748, Fig. 1A,B/HR 339, Fig. 1A,B; emphasis added):

> The arrow on top indicates the gene including the promoter region (5' dashed line) and the terminator region (3' dashed line).

We find that the arrow on top of Kuipers' Fig. 1A, including the dashed line promoter and dashed line terminator regions, corresponds to, or at least approximates, Hofvander's 4964 bp PGBSS gene. We find that the arrow on top of Kuipers' Fig. 1A, excluding the dashed line promoter and dashed line terminator regions, corresponds to the 3.0 kb (3000 b) BamHI-SpeI sequence identified as SUB10 in Kuipers' Fig. 1A including the substantially complete or full length 5'-3' genomic DNA coding region of the GBSS gene (emphasis added):

> For construction pGBA10 and pKGBA10 the 4.2 kb HindIII fragment containing the complete coding region of the GBSS gene (Visser et al. 1989) was subcloned in pUC19 (=SUB10; Fig. 1A). The 3.0 kb BamHI-SpeI fragment of SUB10 was ligated in reversed orientation into digested pBI121S or pPGB-1S, respectively.

(VDX 4, p. 746, col. 2/HR 337, col. 2);

> The full length GBSS cDNA (pGB50, pKGBA50) and genomic DNA (pGBA10, pKGBA10) constructs were all found to be capable of complete inhibition of GBSS gene expression in a higher percentage of transgenic potato clones (Table 1).

(VDX 4, p. 752, col. 1/HR 343, col. 1); and

> For the construction of pGBA10 and pKGBA10 the 3.0 kb HindIII-SpeI fragment containing the complete coding region of the GBSS gene . . . was subcloned in pUC19.

(Appendix B, p. 3, l. 6-7).

Kuipers' 3.0 kb BamHI-SpeI fragment of SUB10 includes the HindIII-SpeI fragment (VDX 4, p. 748, Fig. 1A, SUB10/HR 339, Fig. 1A, SUB10):

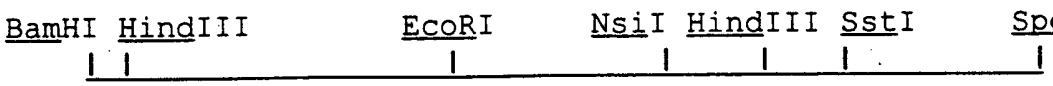
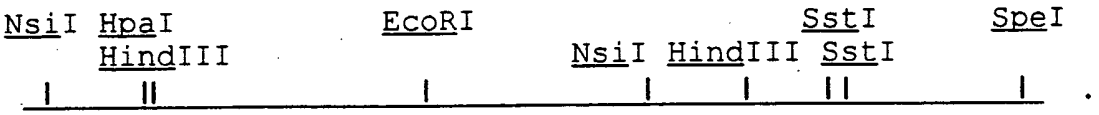


Figure 2 of Hofvander's involved application instructs that the coding region of the GBSS gene depicted below includes a HindIII-SpeI fragment (HR 312 (VDX 10), Fig. 2 "Result of restriction analysis. GBSS coding region including introns are marked in a darker tone."):



VII. We find that the 2961 bp complete genomic DNA sequence Leij depicts at the top of VDX 15/HDX 8, Kuipers' 3.0 kb full length genomic BamHI-SpeI fragment designated SUB10 said to include a HindIII-SpeI subfragment (VDX 4, p. 748, Fig. 1A/ HR 339, Fig. 1A), Visser's 3.0 kb HindIII-SpeI fragment said to contain the complete coding region of the GBSS gene, and the GBSS coding region depicted in Figure 2 of Hofvander's involved application as including a HindIII-SpeI fragment (HR 312/VDX 10), all contain the complete coding region of the GBSS gene.

VIII. We interpret the phrases "full length potato . . . genomic DNA sequence coding for PGBSS" and "full length . . . PGBSS . . . genomic DNA" of the claims of Visser's involved application to mean the complete coding region of the GBSS gene (1) which Kuipers' 3.0 kb BamHI-SpeI fragment SUB10 is said to include (VDX 4, p. 748, Fig. 1A; HR 339, Fig. 1A); (2) by which

Kuipers', Visser's, and Hofvander's HindIII-SpeI fragment is said to be encompassed; (3) to which Leij's 2961 kb sequence substantially corresponds (VDX 15/HDX 8); and (4) from which the antisense constructs pGBA10 and pKGBA10 Visser and Kuipers evaluated (VDX 4, p. 749, Fig. 2A-C/HR 340, Fig. 2A-C) are said to have been made (VDX 4, p. 748, Fig. 1B; HR 339, Fig. 1B). Kuipers observed (VDX 4, p. 749, col. 2; HR 340, col. 2):

> Significant differences were found between the full-length GBSS cDNA and the genomic coding region of the GBSS gene, and between the 35S CaMV promoter and the GBSS promoter. The inhibitory effects of the partial constructs pKGBA25 and pKGBA55 did not differ significantly . . . .

IX. Accordingly, we interpret the phrases "full length potato . . . genomic DNA sequence coding for PGBSS" and "full length . . . PGBSS . . . genomic DNA" in the claims of Visser's involved application as substantially corresponding to Leij's complete 2961 bp coding region of the GBSS gene and the coding region of the GBSS gene including one or more of the HindIII-SpeI fragments depicted in Figure 2 of Hofvander's involved application (HR 312/VDX 10), Figure 2A of Visser's Declaration Under § 132 (Appendix B, last page), and Kuipers' Figure 1A (VDX 4, p. 748).

X. We conclude that the "full length potato . . . genomic DNA sequence coding for PGBSS" and "full length . . . PGBSS . . . genomic DNA" sequences of the antisense constructs of the claims

of Visser's involved application include a HindIII-SpeI fragment of approximately 2961 bp in length and a major fraction of the 1000-2000 bp segment, the entire 2000-3000 bp segment, the entire 3000-4000 bp segment, and a minor fraction of the 4000-4964 bp segment of the 0-4964 bp PGBSS gene depicted in VDX 15 and HDX 8.

XI. We conclude that the DNA fragment of the 0-4964 bp PGBSS gene which Hofvander identifies as SEQ ID No. 1 in the claims of Hofvander's involved application, depicts in HDX 8 and HR 312, and is said either to have inserted or designated for insertion in the genome of a potato plant in antisense direction, is 342 bp in length and includes a minor fraction of the 0-1000 bp segment and a minor fraction of the 1000-2000 bp segment of the 0-4964 bp PGBSS gene depicted in VDX 15 and HDX 8. As best we can determine, the 3' end of the 342 bp fragment (Hofvander's Antisense fragment I) ends where the HindIII-SpeI fragment of the coding region of the PGBSS gene begins (VDX 10 and 2; HR 312 and HDX 8). The specification of Hofvander's involved application discloses (HR 282, l. 29-35):

> The restriction of the pSw with Nsil and HindIII gives fragment I (SEQ ID No. 1) which subcloned in pUC19 is called 19NH35. Further restriction of 19NH35 with HpaI-SstI gives a fragment containing 342 bp of the GBSS gene according to the invention. This fragment comprises leader sequence, translation start and the first 125 bp of the coding region.

XII. We conclude that the DNA fragment of the 0-4964 bp PGBSS gene which Hofvander identifies as SEQ ID No. 3 in the

claims of Hofvander's involved application, depicts in HDX 8 and HR 312, and is said either to have inserted or designated for insertion in the genome of a potato plant in antisense direction, is 492 bp in length and includes a minor fraction of the 3000-4000 bp segment and a minor fraction of the 4000-4964 bp segment of the 0-4964 bp PGBSS gene depicted in VDX 15 and HDX 8. The 5' end of the 492 bp fragment (Hofvander's Antisense fragment III) comprises a minor fraction of the HindIII-SpeI fragment of the coding region of the PGBSS gene including the SpeI end of the HindIII-SpeI fragment (VDX 10 and 2; HR 312 and HDX 8). As best we can determine, the minor fraction of the HindIII-SpeI fragment of the coding region of the PGBSS gene including the SpeI end thereof is the SstI-SpeI subfragment of the HindIII-SpeI fragment of the coding region of the PGBSS gene. The specification of Hofvander's involved application discloses (HR 283, l. 8-14):

> The restriction of pSx with SstI and SpeI gives fragment III (SEQ ID No. 3) which subcloned in pBluescript . . . is called pBlue3'. Further restriction of pBlue3' with BamHI-SstI gives a fragment containing 492 bp of the GBSS gene according to the invention. This fragment comprises the last intron and exon, translation end and 278 bp of trailer sequence.

XIII. We find that Hofvander's 342 bp SEQ ID No. 1 and 492 bp SEQ ID No. 3 do not overlap. In the PGBSS gene, these sequences are separated by a sequence having at least 2000 bp which includes a major fraction of the 1000-2000 bp segment, the entire 2000-3000 bp segment, and a major fraction of the

3000-4000 bp segment of the coding region of the PGBSS gene, which constitute a major fraction of the HindIII-SpeI fragment of the coding region of the PGBSS gene (VDX 10 and 2; HR 312 and HDX 8).

XIV. We conclude that the DNA fragment of the 0-4964 bp PGBSS gene which Hofvander identifies as SEQ ID No. 2 in the claims of Hofvander's involved application, depicts in HDX 8 and HR 312, and either inserted or designated for insertion in the genome of a potato plant in antisense direction, is 2549 bp in length and includes a major fraction of the 1000-2000 bp segment, the entire 2000-3000 segment, and a major fraction of the 3000-4000 bp segment of the 0-4964 bp PGBSS gene depicted in VDX 15 and HDX 8. As best we can determine, the 5' end of the 2549 bp fragment (Hofvander's Antisense fragment II) starts at the 5' HindIII end of the HindIII-SpeI fragment of the coding region of the PGBSS gene or at the HpaI restriction site just before the 5' HindIII end of the HindIII-SpeI fragment of the coding region of the PGBSS gene and ends before the 3' SpeI end of the HindIII-SpeI fragment of the coding region of the PGBSS gene (VDX 10 and 2; HR 312 and HDX 8). As best we can determine, the 2549 bp fragment includes a major fraction of the HindIII-SpeI fragment of the coding region of the PGBSS gene but excludes the SstI-SpeI fragment at the 3' SpeI end of the HindIII-SpeI fragment of the coding region of the PGBSS gene (VDX 10 and 2; HR 312 and HDX 8).

XV. As best we can determine, Hofvander's 2549 bp SEQ ID No. 2 and Hofvander's 342 bp SEQ ID No. 1 either do not overlap at all or overlap over a common HpaI-HindIII segment which is not common to the HindIII-SpeI fragment of the coding region of the PGBSS gene (VDX 10 and 2; HR 312 and HDX 8). The specification of Hofvander's involved application discloses (HR 283, l. 1-7):

> The restriction of pSm with HpaI and NsiI gives fragment II (SEQ ID No. 2) which subcloned in pJRD184 . . . is called pJRDmitt. Further restriction of pJRDmitt with HpaI-SstI gives a fragment containing 2549 bp of the GBSS gene according to the invention. This fragment comprises exons and introns from the middle of the gene.

XVI. As best we can determine, Hofvander's 2549 bp SEQ ID No. 2 and Hofvander's 492 bp SEQ ID No. 3 do not overlap (VDX 10 and 2; HR 312 and HDX 8). Again, see the disclosure in the specification of Hofvander's involved application (HR 283, l. 1-14).

XVII. We conclude that the "full length potato . . . cDNA . . . sequence coding for PGBSS" and the "full length . . . PGBSS . . . cDNA" sequence of the antisense constructs to which the claims of Visser's involved application refer are DNA sequences copied by enzymes from the total mRNA transcripts of the PGBSS gene which complement the "full length . . . PGBSS . . . genomic DNA sequence coding for PGBSS" and "full length . . . PGBSS . . . genomic DNA" sequence of the antisense

constructs of the claims.[11] See pages 11-14 (VR 149-152) and Figure 1 (VR 182) of the specification of Visser's involved application and the references of record cited thereat.

B. Anticipation (35 U.S.C. § 102)

To make a case for no interference-in-fact, Visser must show that no claim in Visser's's involved application which is designated as corresponding to the count defines the same patentable invention as a claim in Hofvander's involved application which is designated as corresponding to the count. 37 CFR § 1.601(j). Accordingly, to show that no claim in Visser's's involved application which is designated as corresponding to the count defines the same patentable invention as a claim in Hofvander's involved application, Visser must show that Visser's claims designated as corresponding to the count are directed to separate patentable inventions from Hofvander's claims designated as corresponding to the count. 37 CFR § 1.601(n). More specifically, if the preponderance of the evidence of record shows either that the subject matter defined by Visser's claims designated as corresponding to the count is not anticipated (35 U.S.C. § 102) by, or obvious (35 U.S.C. § 103) in view of, the subject matter defined by Hofvander's

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[11] cDNA (complementary DNA) is defined as DNA copied from an mRNA molecule by enzymes. Darnell, J. et al., Molecular Cell Biology, Second Edition, W.H. Freeman and Co., New York, N. Y., pp. 214-217 (1990)(Appendix C).

claims designated as corresponding to the count, or the subject matter defined by Hofvander's claims designated as corresponding to the count is not anticipated (35 U.S.C. § 102) by, or obvious (35 U.S.C. § 103) in view of, the subject matter defined by Visser's's claims designated as corresponding to the count, there is no interference-in-fact in this case.

To establish that the subject matter Visser claims is not anticipated by the subject matter Hofvander claims, the evidence as a whole must support a finding that at least one element of the invention Visser claims is not described by Hofvander's claims. In re Spada, 911 F.2d 705, 708, 15 USPQ2d 1655, 1657 (Fed. Cir. 1990)("anticipation or lack of novelty requires . . . that all the elements of the claimed invention be described in a single reference"). "[A]nticipation under § 102 can be found only when the reference discloses exactly what is claimed . . . ." Titanium Metals Corp. v. Banner, 778 F.2d 775, 780, 227 USPQ 773, 777 (Fed. Cir. 1985)(emphasis added).

Visser's claims are directed to gene constructs of the potato plant comprising full length cDNA or genomic DNA coding for PGBSS in reverse orientation, methods of producing transgenic potato plants which comprise integrating a construct comprising full length cDNA or genomic DNA coding for PGBSS in reverse orientation into the genome of the potato plant, and transgenic potato plants produced by integrating a construct comprising full

length cDNA or genomic DNA coding for PGBSS in reverse orientation into the genome of the potato plant. A key element of each claim is a construct comprising full length cDNA or genomic DNA coding for PGBSS in reverse orientation. If the subject matter of Hofvander claims designated as corresponding to the count do not include a construct comprising full length cDNA or genomic DNA coding for PGBSS in reverse orientation for integration into the genome of the potato plant, a step of integrating a construct comprising full length cDNA or genomic DNA coding for PGBSS in reverse orientation into the genome of the potato plant, or a transgenic potato plant genetically engineered by integration of a construct comprising full length cDNA or genomic DNA coding for PGBSS in reverse orientation into the genome of the potato plant, Hofvander's claims designated as corresponding to the count do not anticipate Visser's claims designated as corresponding to the count. If the subject matter of Visser claims designated as corresponding to the count do not include a construct comprising a fragment selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2, or SEQ ID No. 3 in reverse orientation for integration into the genome of the potato plant, a step of integrating a construct comprising a fragment selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2, or SEQ ID No. 3 in reverse orientation into the genome of the potato plant, or a transgenic potato plant genetically engineered

by integration of a construct comprising a fragment selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2, or SEQ ID No. 3 in reverse orientation into the genome of the potato plant, Visser's claims designated as corresponding to the count do not anticipate Hofvander's claims designated as corresponding to the count. For the subject matter defined by one party's designated claims to be anticipated by subject matter defined by the other party's designated claims in this case, the constructs each party's claims for insertion into the genome of the potato plant must be exactly the same. For the constructs defined by one party's designated claims to be exactly the same as the constructs defined by the other party's designated claims in this case, the DNA in reverse orientation in the constructs of each party's claims must be exactly the same. In this case, the evidence as a whole establishes that the DNA in reverse orientation of the constructs defined by the Hofvander's claims designated as corresponding to the count is not exactly the same as the DNA in reverse orientation of the constructs defined by the Visser's claims designated as corresponding to the count.

"A gene is a chemical compound, albeit a complex one . . . ." Amgen Inc. v. Chugai Pharm. Co., 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir.), cert. denied, 502 U.S. 856 (1991). Considering all the evidence of record, the court concluded in Amgen Inc. v. Chugai Pharm. Co., 927 F.2d at 1209,

18 USPQ2d at 1023, "[I]f the DNA sequence was not obvious, host cells containing such sequence . . . could not have been obvious." Based on the evidence in this case, we conclude not only that transgenic potato plants containing constructs comprising a DNA sequence inserted in the genome of the potato plants in reverse orientation would not have been obvious if the DNA sequence itself would not have been obvious but also that methods of producing transgenic potato plants by integrating constructs comprising the DNA sequence into the genome of the potato plants in reverse orientation would not have been obvious if the DNA sequence to be inserted into the genome of the potato plant would not have been obvious. Compare In re Ochiai, 71 F.3d 1565, 1572, 37 USPQ2d 1127, 1133 (Fed. Cir. 1995):

> Having compared Ochiai's claims, limited as they are to the use of a particular nonobvious starting material for making a particular nonobvious end product, to the prior art of record, we reverse . . . .

Subject matter which would not have been obvious, cannot have been anticipated. Structural Rubber Prods. Co. v. Park Rubber Co., 749 F.2d 707, 716, 223 USPQ 1264, 1271 (Fed. Cir. 1984):

> [A] disclosure that anticipates under § 102 also renders the claim invalid under § 103, for "anticipation is the epitome of obviousness," In re Fracalossi, 681 F.2d 792, [794,] 215 USPQ 569[, 571] (CCPA 1982).

Preliminarily, we find that the DNA sequences of the antisense constructs defined in Hofvander's claims are all genomic DNA fragments of the PGBSS gene. Although we find that

full length PGBSS cDNA copied from mRNA transcripts of PGBSS genomic DNA includes ligated fragments of full length PGBSS genomic DNA, we find that full length PGBSS cDNA is not itself a genomic DNA fragment. None of Hofvander's claims define or otherwise describe full length PGBSS cDNA or a DNA sequence copied from an mRNA transcript of the PGBSS gene which is complementary to full length PGBSS genomic DNA.

Next, we repeat our previous conclusion that the "gene construct comprising a fragment of the potato gene which codes [or coding] for [formation of] granule-bound starch synthase [(GBSS) . . .] inserted in the anti-sense direction . . . selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3" (Hofvander's Claims 1, 7, 10 and 50) does not read on, and is not encompassed by claims directed to, a gene construct comprising a fragment of the potato gene coding for GBSS inserted in the anti-sense direction selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3 as a subfragment of a larger fragment of the potato gene coding for GBSS inserted in the antisense direction. Accordingly, we find that the constructs Hofvander claims comprising a fragment of a full length genomic DNA sequence inserted in the antisense direction is not encompassed by, and most certainly does not anticipate, the constructs Visser claims comprising a full length genomic DNA sequence inserted in the antisense direction. As we

interpret the subject matter the parties to this interference claim, inserts comprising a DNA fragment of a DNA segment of the PGBSS gene in the antisense direction do not read on, or anticipate, inserts comprising the DNA segment of the PGBSS gene in the antisense direction. As we interpret the subject matter the parties to this interference claim, inserts comprising a DNA segment of the PGBSS gene in the antisense direction also do not read on, or anticipate, inserts comprising a DNA fragment of a DNA segment of the PGBSS gene in the antisense direction.

Having considered all the evidence in this case, we find that none of Hofvander's 342 bp SEQ ID No. 1 which excludes substantially all of the HindIII-SpeI segment of the coding region of the PGBSS gene, Hofvander's 2549 bp SEQ ID NO. 2 which includes all but the SstI-SpeI segment of the HindIII-SpeI segment of the coding region of the PGBSS gene, and 492 bp SEQ ID No. 3 which includes no more than the SstI-SpeI segment of the HindIII-SpeI segment of the coding region of the PGBSS gene, anticipates the Visser's full length PGBSS genomic DNA sequence coding for PGBSS including all of the HindIII-SpeI segment of the coding region of the PGBSS gene.

In this case, less complex chemical codes inserted in the potato genome in the antisense direction do not anticipate more complex chemical codes inserted in the potato genome in the

antisense direction, and vice versa. On their face, Hofvander's claims do not anticipate Visser's claims, and vice versa.

Nevertheless, Hofvander argues that the antisense constructs, transgenic potatoes, and methods or making and using the same it describes and claims are identical to the constructs, transgenic potatoes, and methods of making and using the same Visser describes and claims because, irrespective of the number and kind of base pairs in the distinct DNA sequences inserted into the potato genome in the antisense direction, once inserted in the antisense direction, Hofvander's fragments and Visser's sequences identically inhibit expression of PGBSS in potato plants and thus are functionally and practically the same. In our view, Hofvander's argument has no merit because the claims of the respective parties recite DNA fragments and/or segments in the antisense direction and constructs comprising and/or containing DNA fragments and/or segments in the antisense direction which are defined by distinct chemical structures in addition to their function. Accordingly, that the different chemical components Hofvander and Visser employ may function identically when inserted into the genome of potato plants in the antisense direction because of one or more common DNA segments, chemical characteristics, etc., is irrelevant. The chemical inserts are not the same. Moreover, the evidence of record does not support the proposition that the respective DNA fragments

and/or segments have a common chemical characteristic or structure which functions to substantially inhibit PGBSS when inserted into the genome of potato plants in the antisense direction. We take particular note that Hofvander's constructs include three fragments of the potato gene (1) at least one of which has no common DNA sequences with Visser's full length PGBSS genomic DNA, and (2) no two of which themselves have a common DNA sequence.

C. Obviousness (35 U.S.C. § 103)

We assume that each party's claims are prior art with respect to the other party's claims. 37 CFR §§ 1.601(j) and 1.601(n). Assuming first that Hofvander's claims designated as corresponding to the count are prior art with respect to Visser's claims designated as corresponding to the count, the consistent criterion for determining obviousness under 35 U.S.C. § 103 is whether the prior art would have led persons having ordinary skill in the art to make and use subject matter Visser claims with reasonable expectation of success. In re Dow Chem. Co., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). "For obviousness under § 103, all that is required is reasonable expectation of success." In re O'Farrell, 853 F.2d 894, 904, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988). A case for obviousness is not established where the prior art would have led persons having ordinary skill in the art to explore a new technology or general

approach that seemed to be a promising field of experimentation but provided no more than general guidance as to the particular form of the claimed invention or how to achieve it. In re O'Farrell, 853 F.2d at 903, 7 USPQ2d at 1681.

We direct the parties' attention to the Background section of Enzo Biochem. Inc. v. Calgene Inc., 188 F.3d 1362, 1366-1369, 52 USPQ2d 1129, 1131-1133 (Fed. Cir. 1999), for its general description of the state of antisense technology from about 1990 to 1992. Most especially consider the following footnote at 1367 n. 4, 52 USPQ2d at 1132 n. 4 (emphasis added):

> Although there is no universally agreed-upon mechanism for the manner in which antisense works to block gene expression in a cell, . . . [Figure 2 at 1367, 52 USPQ2d at 1132,] presents one possible mechanism.

In Enzo Biochem. Inc. v. Calgene Inc., 188 F.3d at 1368, 52 USPQ2d at 1133, the court considered the following representative cell, method, and construct claims:

> (1) A prokaryotic or eukaryotic cell containing a non-native DNA construct, which construct produces an RNA which regulates the function of a gene, said DNA construct containing the following operably linked DNA segments:
>
> (A) a transcriptional promoter segment;
> (B) a transcription termination segment;
> and therebetween
> (C) a DNA segment;
>
> whereby transcription of the DNA segment produces a ribonucleotide sequence which does not naturally occur in the cell, is complementary to a ribonucleotide sequence transcribed from said gene, and said non-

naturally occurring ribonucleotide sequence regulates the function of the gene.

3. A method of regulating the function of a gene in a prokaryotic or eukaryotic cell which comprises introducing into said cell the DNA construct of claim 1.

5. A non-native DNA construct which, when present in a prokaryotic or eukaryotic cell containing a gene, produces an RNA which regulates the function of said gene, said DNA construct containing the following operably linked DNA segments:

a. a transcriptional promoter segment;
b. a transcription termination segment; and
c. a DNA segment comprising a segment of said gene, said gene segment located between said promoter segment and said termination segment and being inverted with respect to said promoter segment and said termination segment, whereby the RNA produced by transcription of the inverted gene segment regulates the function of said gene.

In review of a district court's findings relative to the level of predictability/unpredictability in the art from about 1990 to about 1992, the court stated, Enzo Biochem. Inc. v. Calgene Inc., 188 F.3d at 1372, 52 USPQ2d at 1136:

> The district court next found that antisense was a highly unpredictable technology, a finding amply supported by the record. See, e.g., Inventor Inouye Test., J.A. at 349 (analogizing the predictability of antisense to "drilling for oil"); Calgene Expert Douglas A. Melton, Ph.D. Dep. J.A. at A26,884 ("[T]his method is not universally applicable, it hasn't proven to be, and that's why it's such an interesting area of research, because scientists don't understand the rules."). A text on cell biology, which was introduced into evidence at trial by Enzo, made the observation that:

> It is, however, important to realize that antisense strategies have not been universally straightforward or as easy to apply as was initially hoped, nor has the interpretation of results always been unambiguous, and this has perhaps led to their premature dismissal in certain instances.

11 "Antisense RNA and DNA" in Modern Cell Biology 3 (James A.H. Murray ed. 1992). Based on the evidence before the district court, we conclude that the court also did not err in finding that antisense technology was highly unpredictable.

The court added, Enzo Biochem. Inc. v. Calgene Inc., 188 F.3d at 1375, 52 USPQ2d at 1139:

> Calgene noted, if Calgene were able to explain why antisense could not be applied in a reproducible fashion, that by itself would have been a "groundbreaking scientific discovery" . . . .

The court also concluded that "the district court did not clearly err in finding that the quantity of experimentation required to practice antisense was quite high." Id. at 1374, 52 USPQ2d at 1138.

Nevertheless, this is a different case with different evidence and other facts. Notwithstanding its findings with regard to predictability at the time, the Enzo court noted, "In view of the rapid advances in science, we recognize that what may be unpredictable at one point in time may become predictable at a later time." Id. at 1374 n. 10, 52 USPQ2d at 1138 n. 10. Moreover, the question of predictability in this case does not require us to consider whether the specifications of the respective parties' involved applications would have enabled one

skilled in the art at the time to make and use the full scope of the subject matter claimed without undue experimentation. The issue in this case is not whether persons having ordinary skill in the art at the time reasonably would have predicted success using antisense technology generally to regulate the function of a wide variety of genes in prokaryots and eukaryots as was claimed in the Enzo case based on evidence of success using the limited kinds and number of antisense constructs shown in the Enzo case to regulate the function of particular genes in E. coli or tomato plant cells. In this case, we are asked to consider whether persons having ordinary skill in the art reasonably would have expected that the function of the potato GBSS gene could be regulated in potato plants by inserting a gene construct comprising Visser's full length cDNA or genomic DNA coding for PGBSS into the genome of the potato plant based on evidence that the function of the potato GBSS gene could be regulated in a potato plant by inserting a genomic DNA fragment selected from Hofvander's group consisting of three specifically identified fragments of the PGBSS gene, including DNA segments found inside and/or outside the coding region of the PGBSS gene, into the genome of the potato plant. Given the findings in Enzo Biochem. Inc. v. Calgene Inc., supra, we are not convinced that any evidence in this record of prior successes and/or failures using antisense technology to regulate the function of other genes in

other prokaryotic or eukaryotic species is material to the antisense technology of this interference which is specifically designed to regulate the function of the PGBSS gene in potato plants.

In this case, the burden to establish the level of predictability in the art initially sits with Visser. Having considered and weighed all the evidence of record, we find that the preponderance of the evidence of record establishes that antisense technology, even as limited to the subject matter of the parties' claims designated as corresponding to the interference count in this case, was highly unpredictable at the time the parties made their inventions.

The specification of Visser's involved application states (VR 144, l. 7-11):

> Visser (1989)[12] tested whether the antisense approach could be used to inhibit the expression of the gene for granule-bound starch synthase in potato using heterologous antisense constructs, i.e., an antisense gene constructed from a maize genomic GBSS gene.

According to Visser's specification, the "results were not too encouraging" (VR 147, l. 6). Hofvander does not appear to disagree with Visser's analysis of Visser's 1989 experiments.

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[12] Visser (Visser's PhD Thesis), "Manipulation of the Starch Composition of *Solanum Tuberosum* L. Using *Agrobacterium Rhizogenes* Mediated Transformation," PhD Thesis, University of Groningen, The Netherlands, pp. 9-139 (February 27, 1989)(VDX 7)

Aside from its own work, Visser considers Hergersberg[13] (VDX 1) and Leij (VDX 3) to be the closest prior art to the subject matter claimed in Hofvander's involved application (VB 20). Leij discloses the complete 2961 bp genomic nucleotide sequence of the PGBSS gene (VDX 3, p. 243, Fig. 1; HDX 8; VDX 15), but Leij was not concerned with antisense technology. With regard to the patentability of the subject matter Hofvander claims and the subject matter of Visser's own claims designated as corresponding to the count in view of Hergersberg's teachings, the APJ's decision on preliminary motions states (Paper No. 74, p. 6):

> With respect to the Hergersberg publication, the APJ agrees with Hofvander that this publication would not render the Hofvander claims unpatentable. The Hergersberg antisense sequences, assuming that the sequences are antisense, are much smaller than those used by Hofvander. When the Hergersberg antisense sequences are incorporated into a potato plant, the modified plant reduced amylose production by 30%. Since a potato normally produces amylose in an amount of 20 to 25%, it would appear that Hergersberg's modified potato plants produced amylose in an amount of from 14% to 18%, whereas Hofvander's modified potato plants result in production of 6 to 9% amylose. Moreover, in distinguishing over the Hergersberg publication, the Hofvander opposition . . . also relies upon the same reasons as did Visser in urging that his claims were patentable over this publication.

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[13] Hergersberg, "A Molecular Analysis of the *waxy* Gene from Solanum tuberosum and Expression of waxy antisense RNA in transgenic Potatoes," Inaugural-Dissertation zur Erlangung des Doktorgrades der Mathematisch-Naturwissenschaftlichen Fakultat der Universitat zu Koln, University of Cologne, Cologne, pp. 1-79 (1988) (VDX 1)

Visser maintains the view that the subject matter of its claims designated as corresponding to the count is patentable over Hergersberg's teachings (VB 25, second full para.). However, Visser argues that the decision on preliminary motions holding Hofvander's claims designated as corresponding to the count patentable over Hergersberg's teachings is erroneous because the APJ was not fully apprized of Hergersberg's teachings (VB 21). Visser argues (VB 21; footnote included in text):

> Although the APJ was advised that Hergersberg discloses a GBSS cDNA fragment of about 275 base pairs, the APJ was not advised that another much longer fragment was used in Hergersberg's antisense constructs. Further, the APJ was not advised that Hergersberg used both of these fragments in a single antisense construct.
>
> Several schematic diagrams comparing the sequences used by each of Hofvander, Visser and Hergersberg, namely, VDX2, HDX8 and VDX15 [n. "HDX8 and VDX15 are "corrected" versions of VDX2."], were presented during the motions period; however, none of them accurately depict the antisense fragments used by Hergersberg. . . . .

Providing what Visser considers to be a clearer view of Hergersberg's disclosure, Visser states (VB 22, second para., through VB 22-23, bridging para.)(footnote included in text):

> Although [the APJ's statement that "[t]he Hergersberg antisense sequences . . . are much smaller than those used by Hofvander" (Paper No. 74, p. 6)] . . . may be correct for the 275 base pair fragment when compared to SEQ ID No. 2 of Hofvander, clearly the 275 base pair fragment is not much smaller than Hofvander fragment 1 (SEQ ID No. 1) depicted as a 342 base pair fragment ("nr. 1") in HDX8 and VDX15. In addition, it is now clear that fragment 1 of Hofvander overlaps with Hergersberg's 275 base pair fragment by about 240 base pairs (approximately 87%)(VDX29, HR139, 155-156).

> In any event, there is a much larger fragment disclosed in Hergersberg. This fragment is about 800 base pairs long and falls wholly within fragment 2 (SEQ ID No. 2) of Hofvander. This is confirmed by the testimony of Rask (HR140-142, 156). The about 800 base pair fragment is shorter than only one of Hofvander's antisense sequences, namely, fragment 2 (SEQ ID No. 2). Furthermore, Hergersberg uses both the 275 base pair fragment and the about 800 base pair fragment in a single construct [n. "Although Bruinenberg refers to the fragment as being approximately 500 base pairs long, it is the same fragment which Rask refers to in his testimony as an 800 base pair fragment (HDX38)"]. . . . .

Visser further defines the antisense sequences Hergersberg utilized to produce its constructs as follows (VB 24, last para.; emphasis added):

> As mentioned above, Hergersberg teaches two antisense GBSS <u>cDNA fragments</u> which are used in several different constructs. It is now clear from the record that the 275 base pair fragment of Hergersberg overlaps with fragment 1 (SEQ ID No. 1) of Hofvander, and the 800 base pair fragment falls wholly within fragment 2 (SEQ ID No. 2) of Hofvander (HR139-142, 155-156).

Even accepting all Visser's arguments as correctly representing the facts indicated therein, we still require a reasonable explanation why it would have been obvious to persons having ordinary skill in the art to insert Hofvander's 342 bp genomic DNA fragment (SEQ ID No. 1), Hofvander's 2549 bp genomic DNA fragment (SEQ ID No. 2), or Hofvander's 492 bp genomic DNA fragment (SEQ ID No. 3) in the antisense direction into the genome of potato plants to regulate expression of the PGBSS gene in view of Hergersberg's instructions to insert its 275 bp cDNA fragment and/or 500/800 bp cDNA fragment in the antisense

direction into the genome of a potato plant to regulate expression of the potato GBSS gene, yet it would not have been obvious to persons having ordinary skill in the art in view of the subject matter Hofvander claims to insert Visser's full length cDNA or genomic DNA sequence coding for potato GBSS in the antisense direction into the genome of potato plants to regulate expression of the potato GBSS gene.

Without acknowledging that the subject matter it claims would have been prima facie obvious in view of Hergersberg's teachings, Visser argues that "Visser's claims are patentable over Hergersberg, inter alia, because Visser has achieved an unexpected result" (VB 25). Faced with Visser's main arguments that (1) no interference-in-fact exists between its claimed constructs comprising full length cDNA or genomic DNA sequence coding for potato GBSS in the antisense direction and Hofvander's claimed constructs comprising a fragment selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 or SEQ ID No. 3 in the antisense direction, and (2) the subject matter Hofvander claims would have been obvious in view of Hergersberg's teaching, we first consider whether Hofvander's claims would have been prima facie obvious in view of Hergersberg's teaching.

The evidence of record appears to show that Hofvander's 342 bp SEQ ID No. 1 is a genomic DNA fragment of the promoter region of the GBSS gene found primarily outside the coding region

of the GBSS gene, i.e., it is not a fragment of Visser's full length cDNA or genomic DNA sequence coding for GBSS. According to Visser, the evidence of record shows that Hergersberg's 275 bp cDNA overlaps Hofvander's 342 bp SEQ ID No. 1, i.e., it is cDNA corresponding to a genomic DNA fragment of the GBSS gene found for the most part inside the promoter region and outside the coding region of the GBSS gene. Hergersberg's 275 bp cDNA sequence is not encompassed by either Visser's full length cDNA or genomic DNA sequence coding for GBSS. According to Visser, the evidence of record also shows that Hergersberg's 500/800 bp cDNA fragment in the antisense direction is fully encompassed by Hofvander's 2549 bp SEQ ID 2 in the antisense direction and that both Hergersberg's 500/800 bp fragment and Hofvander's 2549 bp SEQ ID 2 in the antisense direction are fully encompassed either by Visser's full length cDNA or genomic DNA sequence coding for GBSS in the antisense direction. It reasonably would appear from the above that if Hofvander's genomic 342 bp SEQ ID No. 1 in the antisense direction would have been prima facie obvious to persons having ordinary skill in the art in view of Hergersberg's construct comprising its 275 bp cDNA fragment in the antisense direction, which overlaps Hofvander's genomic 342 bp SEQ ID No. 1 in the antisense direction, and if Hofvander's genomic 2549 bp SEQ ID No. 2 in the antisense direction would have been prima facie obvious to persons having ordinary skill in the art in view

of Hergersberg's construct comprising its 500/800 bp cDNA fragment in the antisense direction, which is a subfragment of Hofvander's 2549 bp SEQ ID No. 2 in the antisense direction (VB 26-29), then Visser's constructs comprising full length cDNA or genomic DNA coding for GBSS in the antisense direction also would have been prima facie obvious to persons having ordinary skill in the art in view of either Hofvander's construct comprising its genomic 2549 bp SEQ ID No. 2 in the antisense direction or Hergersberg's construct comprising its 500/800 bp cDNA antisense subfragment of Hofvander's 2549 bp SEQ ID No. 2 antisense fragment of Visser's constructs comprising full length cDNA or genomic DNA coding for GBSS in the antisense direction.

Irrespective of the irreconcilability of conclusions in the decision on preliminary motions, Hofvander maintains that Visser's constructs comprising full length cDNA or genomic DNA sequence coding for GBSS in the antisense direction are the same patentable invention as Hofvander's constructs comprising at least one fragment selected from the group consisting of SEQ ID 1, SEQ ID No. 2 or SEQ ID No. 3 in the antisense direction, but denies that any of its genomic DNA fragments in the antisense direction would have been prima facie obvious for use in regulating potato GBSS gene expression in view of either Hergersberg's construct comprising its 275 bp cDNA fragment in the antisense direction which substantially overlaps Hofvander's

342 bp SEQ ID No. 1 fragment in the antisense direction or Hergersberg's construct comprising its 500/800 bp cDNA fragment in the antisense direction which is completely encompassed by Hofvander's 2549 bp SEQ ID 2 fragment in the antisense direction.

We find Hofvander's explanations why it considers its claims patentable over Hergersberg's teachings, even though they are directed to the same patentable invention as the claims of Visser's involved application, to be somewhat dubious. We are not persuaded that Hofvander's seemingly inconsistent positions are consistent because "Hergersberg fails to teach the antisense constructs of Hofvander's invention and also fails to teach that amylose formation may be effectively suppressed by using antisense technology as claimed by Hofvander" (HB 45, first para.). Visser also fails to teach the antisense constructs of Hofvander's invention and also fails to teach that amylose formation may be effectively suppressed by using antisense technology as claimed by Hofvander.

Next, Hofvander argues that an antisense DNA sequence which is homologous to the potato GBSS gene and functions to inhibit the expression of the potato GBSS gene is present in Visser's full length cDNA, Visser's full length genomic DNA sequence, and each of Hofvander's genomic DNA fragments, and (2) regulates GBSS expression in a potato plant when inserted into its genome (HB 26-32). While the same antisense DNA sequence which is

homologous to the potato GBSS gene and functions to inhibit the expression of the potato GBSS gene not only may be present in Visser's full length cDNA or genomic DNA sequence and Hofvander's 2549 bp genomic DNA fragment and regulate GBSS expression in a potato plant when inserted into its genome, it may also be present in Hergersberg's 500/800 bp cDNA fragment and regulate GBSS expression in a potato plant when inserted into its genome. Nevertheless, Hofvander argues that expert testimonies presented in this record by both parties portray Hergersberg's publication as being so fraught with error that persons having ordinary skill in the art reasonably would not have relied upon Hergersberg's disclosure for DNA fragments capable of regulating potato GBSS gene expression when inserted into the genome of a potato plant in the antisense direction or for any other information (HB 45, first para.). More specifically, Hofvander states (VB 46, final para.):

> Hergersberg is not a reliable reference and would fail to disclose or suggest <u>anything</u> to a person skilled in the art. As stated by Dr. Rask, Hergersberg is not reliable because "Hergersberg's thesis is one of the most sloppy thesis I've ever seen and I'm astonished that it passed if it ever did" [Rask Testimony: HR 000140; page 77, lines 11-14] . . . .

Notwithstanding the expert testimony of record that Hergersberg's presentation was sloppy and showed Hergersberg's propensity for error, we find ample evidence of record that the parties to this interference and their colleagues found

Hergersberg's work reliable enough to support their own experimentation. For example, the specification of Hofvander's involved application states (HR 281, l. 33-37):

> The genomic library has been screen for the potato GBSS gene by means of cDNA clones for both the 5' and 3' end of the gene (said cDNA clones being obtained from M Hergersberg, Max Plank Institute in Cologne) according to a protocol from Clontech.

Both the specification of Visser's involved application (VDX 152) and Visser's 1991 publication (VDX 8, p. 290, bridging para.) disclose:

> Two subclones encompassing a full-length cDNA clone from potato GBSS isolated from lambda NM1149 library (Hergersberg 1988; Visser et al. 1989d) were used as indicated in Fig. 1 for the construction of the antisense and sense binary vectors.

See also acknowledgments to Hergersberg's PhD Thesis in van der Leij's 1991 publication (VDX 3, p. 240, col. 2) and thereafter in Kuipers' 1994 publication (HDX 29, p. 51, col. 1, last para.). Hofvander's criticism of Hergersberg's work might be justified had the art in 1988 attained a level of maturity such that persons having ordinary skill in the art at the time would have considered antisense technology predictable and erroneous experimental procedure and/or results or erroneous reporting of valid experimental procedure or results unacceptable. However, we find that antisense technology at the time Hergersberg's PhD thesis was first published was still in its infant stage. We find that persons having ordinary skill in the art in the time

period from 1990-1992 would have relied on the teaching of Hergersberg's earlier published PhD Thesis at least to the extent persons having ordinary skill in the art can rely on incipient publications in a highly unpredictable art and the art continues to be unpredictable. Accordingly, if the preponderance of the evidence of record indicates that persons having ordinary skill in the art reasonably would have expected that a DNA sequence common to Visser's full length potato GBSS cDNA and genomic DNA sequences in the antisense direction and Hofvander's 2549 bp genomic SEQ ID No. 2 in the antisense direction is responsible for regulating GBSS expression in a potato plant when inserted into its genome (HB 26-32), then the art would have attained at the time a level of predictability which not only would have justified rejections of the subject matter Visser claims as prima facie obvious under 35 U.S.C. § 103 in view of Hofvander's claims, the combined teachings of Hergersberg and van der Leij, or a combination of Hofvander's claims and the teachings of Hergersberg and van der Leij, but also would have justified a rejection of the subject matter Hofvander claims as prima facie obvious under 35 U.S.C. § 103 in view of the combined teachings of Hergersberg and van der Leij. On the other hand, if a preponderance of the evidence of record indicates that the pertinent antisense technology would have continued to be highly unpredictable at the critical time, then we must conclude not

only that the subject matter Hofvander claims would not have been prima facie obvious under 35 U.S.C. § 103 in view of the combined teachings of Hergersberg and van der Leij, but that the subject matter Visser claims would not have been prima facie obvious under 35 U.S.C. § 103 in view of the subject matter Hofvander's claims, the combined teachings of Hergersberg and van der Leij, or a combination of Hofvander's claims and the teachings of Hergersberg and van der Leij, i.e., Hofvander's claims designated as corresponding to the count and Visser's claims designated as corresponding to the count are patentable over Hergersberg's teaching and there is in this case no interference-in-fact.

To the contrary, Hofvander argues that the following "facts" establish a reasonable level of predictability for the particular antisense technology to which the parties claims designated as corresponding to the count relate:

> The important thing about antisense technology is . . . that the DNA fragment (segment) used is homologous to the gene that is to be inhibited and functions to inhibit the expression of that same gene. Full length or not is of no importance.

(HB 27 (citing HB 6, Fact 6));

> [A]s shown by Hofvander, both fragments of and the full length GBSS gene will result in inhibition of amylose formation and thus production of essentially amylose-free amylopectin.

(HB 27-27, bridging para.);

> By reference to the Interference Initial Memorandum, it can be seen that the Examiner considered the use of

> fragments and the use of full length sequence to suppress amylose formation in potato to be the same patentable invention, e.g., the use of full length sequence of the potato GBSS gene for amylose suppression to be obvious in view of the use of fragments of the potato GBSS gene for amylose suppression.

(HB 28, first full para. (citing HB 4, Fact 1));

> The Visser application and the Hofvander application both support the position that the use of full length sequences and the use of fragments in antisense orientation to obtain essentially amylose-free starch define the same patentable invention as the Count of the interference. . . . . The "invention" encompassed by the Count, the priority of which is to be determined in this interference, describes how to produce essentially amylose-free starch in the form of amylopectin by introducing DNA constructs into the genome of a potato. While the Visser application in this interference claims the use of full length sequences to accomplish this objective, the Hofvander application, which has an earlier effective filing date, claims the use of fragments to accomplish this same objective. Because antisense constructs comprising the full length sequence and antisense constructs comprising fragments of the GBSS sequence are functionally equivalent, the same patentable invention is defined by the Hofvander and the Visser claims.

(HB 28, second full para. (citing HB 4-5, Facts 2-4); emphasis added).

As support for its arguments, including the homologous sequence theory presented for the first time in its brief, Hofvander relies on the assertions of experts. However, "[n]othing in the [Federal R]ules [of Evidence] or in . . . [Federal Circuit] jurisprudence requires the fact finder to credit . . . unsupported assertions of an expert witness." Rohm and Haas Co. v. Brotech Corp., 127 F.3d 1089, 1092,

44 USPQ2d 1459, 1462 (Fed. Cir. 1997). Attorney argument unsupported by factual evidence is insufficient to establish the level of predictability in the art. In re Lindner, 457 F.2d 506, 508, 173 USPQ 356, 358 (CCPA 1972).

We presented in an earlier section of this decision the reasons why we cannot conclude that Hofvander's claims designated as corresponding to the count and Visser claims designated as corresponding to the count define the same patentable invention based solely on factual evidence that antisense constructs comprising the full length cDNA or genomic DNA sequence and antisense constructs comprising fragments of the GBSS gene are functionally equivalent when the evidence shows that the chemical structures of the antisense DNA sequences the respective parties utilize to (1) make and use its constructs for insertion into the potato genome to regulate GBSS gene expression in potato plants and formation of transgenic potato plants including said constructs, and (2) carry out the methods of producing transgenic potato plants transformed by said constructs which the parties claim, are neither identical nor structurally obvious over each other. See again In re Dillon, 919 F.2d 688, 694, 16 USPQ2d 1897, 1903 (Fed. Cir. 1990)(en banc), cert. denied, 500 U.S. 904 (1991):

> The materials used in a claimed process as well as the result obtained therefrom must be considered along with the specific nature of the process, and the fact that

> new or old, obvious or nonobvious, materials are used or result from the process are only factors to be considered, rather than conclusive indicators of the obviousness or nonobviousness of the claimed process. When any applicant properly presents and argues suitable method claims, they should be examined in light of all these relevant factors, free from any presumed controlling effect of Durden. Durden did not hold that all methods involving old process steps are obvious; the court in that case . . . refused to adopt an unvarying rule that the fact nonobvious starting materials and nonobvious products are involved ipso facto makes the process nonobvious. Such an invariant rule always leading to the opposite conclusion is also not the law.

Even if persons having ordinary skill in the art would have considered all of Visser's full length potato cDNA and genomic DNA sequences coding for GBSS in the antisense direction and Hofvander's SEQ ID Nos. 1, 2 and 3 in the antisense direction all to be GBSS gene fragments, the evidence of record does not establish that persons having ordinary skill in the art reasonably would have considered any one of the chemical structures of any one of Visser's GBSS gene fragments to be either the same as, or obvious in view of, any one of the chemical structures of any one of Hofvander's GBSS gene fragments, and vice versa. Even if Visser's specification does contemplate using whatever "sufficient part" (VR 149, l. 8) of antisense PGBSS cDNA or genomic DNA sequence may be functionally "effective for obtaining tubers containing amylose-free starch" (VR 149, l. 8-9), i.e., using functionally effective fragments of the full length cDNA or genomic DNA sequence coding for PGBSS in

the antisense direction (VR 149, l. 7-9), we interpret Visser's claims to define no less than the full length cDNA or genomic DNA sequence coding for PGBSS in the antisense direction. Rather than explain why the chemical structures of Visser's fragments in the antisense direction for use in regulating GBSS gene expression in potato plants would have been obvious in view of the chemical structures of Hofvander's fragments in the antisense direction for use in regulating GBSS gene expression in potato plants, Hofvander argues that the comparative chemical structures of the PGBSS gene fragments the respective parties direct to be inserted into the genome of a potato plant are immaterial as long as each of the DNA fragment or fragments the respective parties utilize to regulate PGBSS gene expression "is homologous to the gene that is to be inhibited and functions to inhibit the expression of the same gene" (HB 27, l. 5-6).

The evidence shows that recognition by a person skilled in the art that DNA fragments including one sequence which is homologous to the PGBSS gene to be inhibited in the antisense direction and functions to inhibit the expression of the PGBSS gene reasonably would not have suggested to a person skilled in the art that other DNA fragments including other sequences which are similarly or otherwise homologous to the PGBSS gene to be inhibited in the antisense direction also would function to inhibit the expression of the PGBSS gene or that other DNA

fragments including other sequences not similarly or otherwise homologous to the PGBSS gene to be inhibited would not function to inhibit expression of the PGBSS gene. Hofvander's SEQ ID Nos. 1, 2 and 3 appear not to have a common DNA sequence among them which in the antisense direction could be responsible for regulating PGBSS gene expression. Moreover, while Hofvander teaches that any one of SEQ ID Nos. 1, 2, and 3 functions to inhibit expression of the PGBSS gene, only Hofvander's 2549 bp SEQ ID No. 2 and 492 bp SEQ ID No. 3 in the antisense direction appear to be encompassed by, or overlap, Visser's full length potato cDNA or genomic DNA sequence coding for PGBSS in the antisense direction.

In our view, Kuipers' 1995 publication[14] of record contains evidence which undermines Hofvander's homologous sequence theory. Kuipers' 1995 publication establishes that (1) the antisense technology to which the claims corresponding to the count pertains is highly unpredictable, and (2) Hofvander's claims and Visser's claims designated as corresponding to the count are directed to separate patentable inventions even if the DNA

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[14] Kuipers, et al., "Factors Affecting the Inhibition by Antisense RNA of Granule-Bound Starch Synthase Gene Expression in Potato," Mol. Gen. Genet., Vol. 246, pp. 745-755 (1995)(Kuipers' 1995 publication)(HR 336; VDX 4). A substantial part of Figure 1A,B of Kuipers 1995 publication (HR 339; VDX 4, p. 748) appears in Fig. 2A,B attached to Declaration Under 37 CFR § 1.132 of Richard G. F. Visser (HDX 9) filed Visser's involved application (Paper No. 27 in Visser's Application 08/294,619).

fragments they claim have common sequences which are homologous to the PGBSS gene to be inhibited in the antisense direction.

Table 1 of Kuipers 1995 publication tabulates (VDX 4, p. 748; HR 339):

> Inhibition of granule-bound starch synthase (GBSS) gene expression assessed by iodine staining of (micro-)tuber starch from transgenic 1024-2 clones carrying different antisense constructs. The antisense constructs pGB50, pKGBA50 and pKGBA55 are based on the GBSS cDNA. The other constructs are based on the genomic coding region of the GBSS gene.

Table 1 is reproduced in its entirety below (VDX 4, p. 748, Table 1; HR 339, Table 1):

Construct	Number of transformants	Number of transformants with inhibition		Number of transformants not inhibition[a]
		Complete[a]	Incomplete[a]	
pGBA10	36	1 (3%)	23 (64%)	12 (33%)
pKGBA10	35	1 (3%)	17 (49%)	17 (49%)
pGBA20	36	0	1 (3%)	35 (97%)
pKGBA20	28	0	2 (7%)	26 (93%)
pKGBA25	49	0	6 (12%)	43 (88%)
pGBA30	87	0	0	87 (100%)
pKGBA30	71	0	1 (1%)	70 (99%)
pKGBA31	41	2 (5%)	21 (51%)	18 (44%)
pKGB50	26	3 (12%)	21 (80%)	2 (8%)
pKGBA50	32	8 (25%)	14 (44%)	10 (31%)
pKGBA55	48	2 (4%)	2 (4%)	44 (92%)

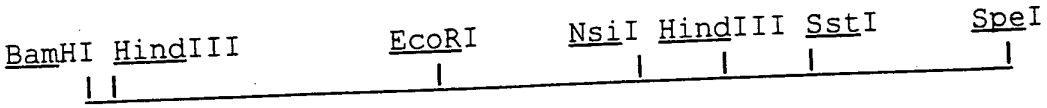
[a] Complete inhibition: starch granules showing red staining starch with a small blue staining core after iodine staining. Incomplete inhibition: starch granules showing a medium sized or large blue staining core and a red staining outer part of the granule after iodine staining. No inhibition: starch granules showing blue staining starch after iodine staining.

Figure 1B defines the constructs indicated in Table 1 as follows (promoter (35S or GB) and either full length genomic DNA coding

for GBSS (SUB10), genomic DNA including fragments of full length genomic DNA coding for GBSS (SUB20, SUB25, SUB30, and SUB31), full length complementary DNA coding for GBSS (GBSS cDNA), or cDNA including fragments of full length complementary DNA coding for GBSS (SUB55) are indicated):

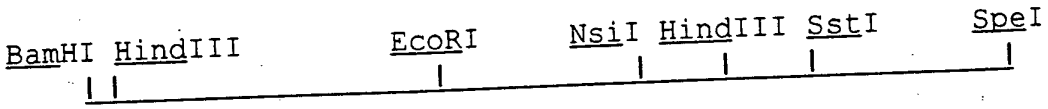
pGBA10 (35S-SUB10)

SUB10



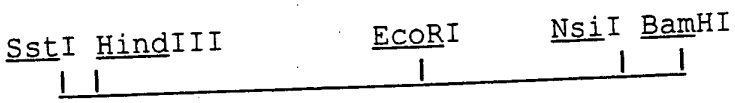
pKGBA10 (GB-SUB10)

SUB10



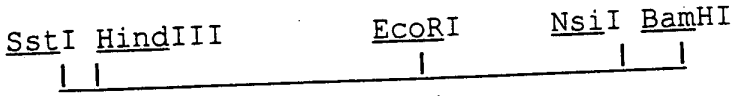
pGBA20 (35S-SUB20)

SUB20



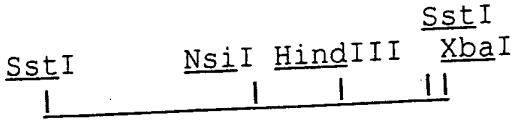
pKGBA20 (GB-SUB20)

SUB20



pKGBA25 (GB-SUB25)

SUB25

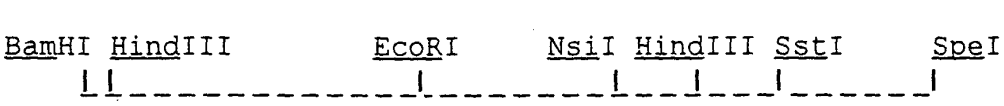


pGBA30 (35S-SUB30)

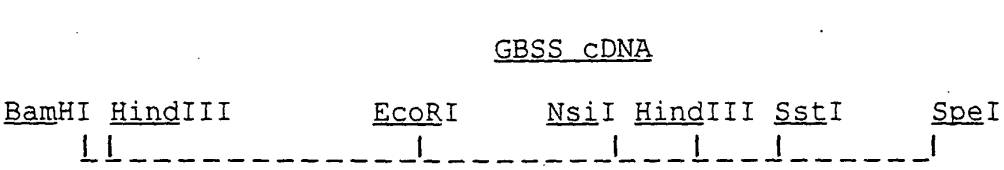
pKGBA30 (GB-SUB30)

pKGBA31 (GB-SUB31)

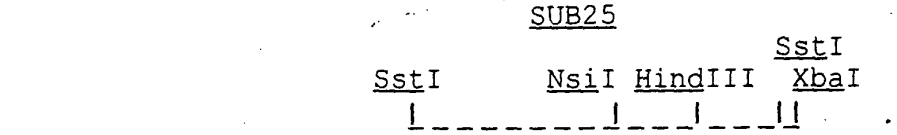
pGB50 (35S-GBSS cDNA)



pKGBA50 (GB-GBSS cDNA)



pKGBA55 (GB-SUB55)[15]



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[15] SUB55 is the cDNA corresponding to gDNA fragment SUB25 (see definition of pKGBA55 in Figure 2B of Kuipers' 1995 publication (HR 340; VDX 4, p. 749).

The Kuipers 1995 publication reported a higher percentage of clones which inhibited PGBSS gene expression using the antisense GBSS cDNA constructs than were inhibited using the corresponding genomic GBSS DNA constructs (VDX 4, p. 752, col. 1; HR 343, col. 1)(emphasis added):

> The origin of the GBSS sequence was shown to be an important factor in determining the efficacy of antisense inhibition. The full-length GBSS cDNA (pGB50, pKGBA50) and genomic DNA (pGBA10, pKGBA10) constructs were all found to be capable of complete inhibition of GBSS gene expression, but it was shown that the antisense GBSS cDNA constructs resulted in complete inhibition of GBSS gene expression in a higher percentage of transgenic potato clones (Table 1). This was also observed for the partial cDNA construct pKGBA55 as compared to the corresponding partial genomic construct pKGB25. The percentage of clones with inhibited GBSS gene expression was shown to be higher for the antisense GBSS cDNA constructs than for the genomic DNA constructs (Fig. 2A). The presence of intron sequences in the genomic constructs might contribute to the observed differences in antisense inhibition. The full length GBSS gene contains 12 introns (van der Leij et al. 1991), four of which are also present in the gene fragment used for pKGBA25. These introns will not be processed when present in antisense orientation. . . . The supposed differences . . . can be explained by the differences in the GC content, which is 42.7% for exon (cDNA) sequences and 33% for intron sequences. . . . In this way, the presence of intron sequences with a low GC content might reduce the efficacy of antisense inhibition of gene expression.

The Kuipers 1995 publication speculates that the presence of intron sequences with a low GC content in the genomic antisense inserts reduces the efficiency of antisense inhibition of GBSS gene expression and results in the differences in antisense inhibition observed. The Kuipers 1995 publication shows that

GBSS gene expression in potato plants is no less inhibited by constructs including cDNA segments in the antisense direction inserted into the potato plant genome than by the corresponding constructs including genomic DNA segments in the antisense direction. However, a higher percentage of potato plants with completely inhibited GBSS gene expression was produced using antisense cDNA inserts than was produced using corresponding antisense genomic DNA inserts.

We fail to understand how Hofvander's homologous sequence theory of obviousness itself explains why Kuipers' pKGBA55 construct, including a cDNA fragment in the antisense direction, a cDNA fragment which corresponds to the SUB25 genomic DNA fragment in the antisense direction used in Kuipers pKGBA25 construct (VDX 4, p. 749, Fig. 2A-C), completely inhibits potato GBSS gene expression while Kuipers' pKGBA25 construct does not (VDX 4, p. 748, Table 1; VDX 4, p. 749, Fig. 2B.). We also fail to understand how Hofvander's homologous sequence theory of obviousness itself explains why Kuipers' pKGBA50 constructs comprising full length cDNA in the antisense direction are 800% more effective in completely inhibiting PGBSS gene expression than Kuipers' pKGBA10 comprising full length genomic DNA in the antisense direction (VDX 4, p. 749, Fig. 2A).

Of greater significance to the issue before us, however, is the following discussion of the experimental results for full

length genomic DNA sequences coding for PGBSS in the antisense direction versus the experimental results for fragments of full length genomic DNA sequences coding for PGBSS in the antisense direction reported in Kuipers' 1995 publication (VDX 4, p. 752, col. 2; emphasis added):

> In transgenic clones, the degree of inhibition of GBSS gene expression was found to vary for the genomic GBSS antisense constructs. However, similar frequencies of complete and incomplete inhibition could be achieved with pGBA10, pKGBA10 and pKGBA31 (comprising 0.6kb of the 3' end of the GBSS coding region and containing one intron sequence). This indicates that the size of the antisense RNA does not affect the efficacy of inhibition. Furthermore, it demonstrates that the GBSS fragment used in pKGBA31, or at least part of it, is essential for the inhibition of GBSS gene expression, as the inhibitory effect of pGBA20, pKGBA20 and pKGBA25 was much lower.
>
> For pGBA30 and pKGBA30, the weak inhibitory effect may be caused by a premature transcription termination. The genomic fragment used for these constructs contains a 3' non-GBSS sequence, which comprises a part of a putative pseudogene (van der Leij et al. 1993), in addition to the GBSS fragment that is also present in pKGBA31. . . . A premature transcription stop does not necessarily result in the absence of antisense inhibition, as has been described for pGB50 (Kuipers et al. 1994) and several other antisense genes . . . but in the case of pGBA30 and pKGBA30 the resulting antisense RNA might lack sequences that are complementary to the GBSS mRNA.

We find from the factual evidence in Table 1 of Kuipers 1995 publication (VDX 4, p. 748, Fig.1A,B and Table 1), Visser's involved application (VR 139+), and Hofvander's involved application (HR 275+, especially HR 312, Fig. 2):

(1) Constructs including Visser's full length genomic DNA coding for PGBSS in the antisense direction are effective for inhibiting PGBSS gene expression in potato plants;

(2) Constructs including Kuipers' SUB10 full length genomic DNA coding for PGBSS in the antisense direction are effective for inhibiting PGBSS gene expression in potato plants;

(3) The coding region of PGBSS depicted in Hofvander's Fig. 2, Visser's full length genomic DNA coding for PGBSS, and Kuipers' SUB10 full length genomic DNA coding for PGBSS, all encompass the same or substantially the same HindIII-SpeI fragment;

(4) The coding region of the PGBSS gene described in Hofvander's Fig. 2 (HR 312) encompasses Hofvander's SEQ ID No. 3;

(5) Kuipers' SUB10 full length genomic DNA sequence coding for PGBSS encompasses Kuipers' SUB31 SstI-SpeI fragment;

(6) Constructs including the SUB31 SstI-SpeI fragment of Kuipers' SUB10 full length genomic DNA sequence coding for PGBSS in the antisense direction are effective for inhibiting PGBSS gene expression in potato plants;

(7) Constructs including Hofvander's SEQ ID No. 3 in the antisense direction are effective for inhibiting PGBSS gene expression in potato plants;

(8) Hofvander's SEQ ID No. 3 in the antisense direction and Kuipers' SUB31 SstI-SpeI fragment in the antisense direction are the same or substantially the same;

(9) Constructs including a Kuipers' SUB30 SstI-BamHI segment in the antisense direction, a segment which encompasses Kuipers' effective SUB31 SstI-SpeI fragment of Kuipers' effective SUB10 full length genomic DNA sequence coding for PGBSS in the antisense direction, are not effective for inhibiting PGBSS gene expression in potato plants;

(10) Constructs including Kuipers' SUB20 SstI-BamHI segment in the antisense direction, a segment which encompasses the HindIII-NsiI fragment of Kuipers' effective SUB10 full length genomic DNA sequence coding for PGBSS in the antisense direction, are not effective for inhibiting PGBSS gene expression in potato plants;

(11) Constructs including Kuipers' SUB25 SstI-XbaI segment in the antisense direction, a segment which encompasses the NsiI-SstI fragment of Kuipers' effective SUB10 full length genomic DNA sequence coding for PGBSS in the antisense direction, are not effective for inhibiting PGBSS gene expression in potato plants;

(12) The coding region of the PGBSS gene described in Hofvander's Fig. 2 (HR 312) encompasses Hofvander's SEQ ID No. 2;

(13) Constructs including Hofvander's SEQ ID No. 2 fragment of the coding region for PGBSS in the antisense direction, a

fragment which encompasses the same or substantially the same HindIII-SstI fragment of Kuipers' effective SUB10 full length genomic DNA sequence coding for PGBSS in the antisense direction and Visser's effective full length genomic DNA sequence coding for PGBSS in the antisense direction, are effective for inhibiting PGBSS gene expression in potato plants; and

(14) Constructs including Hofvander's effective SEQ ID No. 2 fragment of the coding region for PGBAA in the antisense direction not only commonly encompass the same or substantially the same HindIII-SstI fragment encompassed by Kuipers' effective SUB10 full length genomic DNA sequence coding for PGBSS in the antisense direction and Visser's effective full length genomic DNA sequence coding for PGBSS in the antisense direction but also commonly encompass the same or substantially the same HindIII-NsiI fragment encompassed by Kuipers' ineffective SUB20 segment in the antisense direction and Kuipers' ineffective NsiI-SstI fragment included in Kuipers' ineffective SUB25 segment in the antisense direction.

Hofvander does not point to any evidence in this record which reasonably suggests that persons having ordinary skill in the art at the time Visser invented the subject matter defined by its claims designated as corresponding to the count would or could have relied on the homologous sequence theory Hofvander proposes to predict that a full length cDNA or genomic DNA

sequence coding for PGBSS in the antisense direction would be effective to inhibit expression of the GBSS gene in potato plants based on success using a fragment of the full length cDNA or genomic DNA sequence coding for PGBSS in the antisense direction. Nor does Hofvander point to any evidence in this record which reasonably suggests that persons having ordinary skill in the art the time Hofvander invented the subject matter defined by its claims designated as corresponding to the count reasonably would or could have relied on the homologous sequence theory to predict that some fragment of a full length cDNA or genomic DNA sequence coding for PGBSS in the antisense direction would be effective to inhibit expression of the GBSS gene in potato plants based on success using a full length cDNA or genomic DNA sequence coding for PGBSS in the antisense direction to inhibit expression of the GBSS gene in potato plants.

Even if we presume that sequence homology is one factor which persons having ordinary skill in the art unquestionably would have considered in their efforts to successfully apply antisense technology to inhibit GBSS gene expression in potato plants, the evidence shows that sequence homology is but one of many factors which influence success. Kuipers' 1995 publication indicates that persons having ordinary skill in the art reasonably could not have predicted which DNA sequences in the antisense direction would successfully inhibit PGBSS gene

expression based solely on their sequence homology to one or more other DNA sequences which successfully inhibited PGBSS gene expression in the antisense direction.

The Kuipers' 1995 publication provides substantial evidence that the art to which the parties' claims designated as corresponding to the count pertain is influenced by many unforeseeable factors. Kuipers' 1995 publication not only establishes that the pertinent art was highly unpredictable in the 1990-1992 time frame, but it also was unpredictable thereafter. Visser's involved application teaches that Visser's constructs comprising a promoter and full length genomic DNA coding for PGBSS in the antisense direction will inhibit GBSS gene expression in potato plants to the same or substantially the same extent that Hofvander's involved application teaches that Hofvander's constructs comprising the same promoter and SEQ ID No. 3 in the antisense direction, a fragment of Visser's full length genomic DNA coding for PGBSS in the antisense direction will inhibit GBSS gene expression in potato plants. Kuipers 1995 publication thereafter showed that constructs comprising either full length genomic DNA coding for PGBSS or a SstI-SpeI fragment thereof in the antisense direction inhibits PGBSS gene expression. Nevertheless, the later results described in Kuipers' 1995 publication shows that sequence homology alone cannot reasonably be relied upon as an indicator of success using

antisense technology, because the art remains unpredictable. Kuipers' 1995 publication shows that constructs comprising SUB30 in the antisense direction include the same SstI-SpeI sequence in the antisense direction as do effective constructs comprising SUB31 in the antisense direction or effective constructs comprising Visser's full length genomic DNA coding for PGBSS in the antisense direction. Nevertheless constructs comprising SUB30 in the antisense direction do not inhibit PGBSS gene expression while constructs comprising SUB31 and full length genomic DNA coding for PGBSS in the antisense direction fo inhibit PGBSS gene expression. All three DNA sequences include the same homologous SstI-SpeI sequence. Kuipers suspects "premature transcription termination" with antisense SUB30 inserts because "[t]he genomic fragment used for the SUB30 constructs contains a 3' non-GBSS sequence" (VDX 4, p. 752, col. 2, second para.). However, Kuipers also points to prior art which indicates that "[a] premature transcription stop does not necessarily result in the absence of antisense inhibition" (VDX 4, p. 752, col. 2, second para.).

The results in Kuipers' 1995 publication also show that prior knowledge in the art that full length genomic DNA coding for PGBSS in the antisense direction will inhibit PGBSS gene expression reasonably would not have led persons having ordinary skill in the art to expect that similar success would or could be

achieved using any particular fragment thereof in the antisense direction. Compare the successful results using Kuipers' pKGBA10 constructs comprising SUB10 in the antisense direction to the unsuccessful results using Kuipers' pKGBA20 and pKGBA25 constructs respectively comprising SUB20 and SUB25 genomic DNA fragments of SUB10 full length genomic DNA coding for PGBSS in the antisense direction.

Long after the effective filing dates of Hofvander's and Visser's involved applications, Kuipers' 1995 publication reported that, while the knowledge of persons skilled in the art of antisense technology had greatly increased since 1988, still (VDX 4, pp. 752-754 [sic 753], bridging para.):

> . . . variation[s] in the inhibitory effects of the partial genomic antisense constructs [can no more than] point . . . towards a function for certain regions of the gene in antisense inhibition.

According to Kuipers' 1995 publication, most recently reported studies in the art "<u>might indicate</u> that certain sequence characteristics are involved in the process of antisense inhibition" (VDX 4, p. 752, col. 2, final incomplete para.; emphasis added). On the other hand, they might not (VDX 4, pp. 752-754 [sic 753], bridging para.). Alas, Kuipers' 1995 publication points to evidence that "might indicate, that in contrast to what has often been hypothesized" (VDX 4, p. 754 [sic 753], col. 1, last sentence of the first incomplete para.),

other factors are involved. Having considered the evidence in the art up to its submission for publication, Kuipers' 1995 publication generally concludes that "antisense RNA-mediated inhibition of the expression of the GBSS gene offers good prospects for the production of amylose-free tuber starch in potato cultivars" (VDX 4, p. 754 [sic 753], col. 1, last para., last sentence; emphasis added).

Here, as in In re Dow Chem. Co., 837 F.2d at 473, 5 USPQ2d at 1532:

> There must be a reason or suggestion in the art for selecting the procedure [employing the DNA sequence in the antisense direction that the other party] used, other than the knowledge learned from the . . . [other party's] disclosure. . . . . Of the many scientific publications cited . . . none suggests that any [other] process could be used successfully . . . to produce this product having the desired properties.

Absent any reason or suggestion in the prior art to use the constructs comprising the DNA sequences of distinct chemical structure in the antisense direction which the other party inserted into the genome of a potato plant to inhibit PGBSS gene expression in the potato plant with reasonable expectation of success, the claimed inventions of each party to this interference remain prima facie separately patentable over the claimed inventions of the other party to this interference based on the patentably distinct chemical structures of the DNA

sequences each party's claims describe for insertion into the genome of the potato plant in the antisense direction.

Visser has established at least that the parties' claims prima facie are drawn to separate patentable inventions based on their comparatively separate and distinct chemical structures. Thus, the burden has been shifted to Hofvander to show that the inventions the parties claim are directed to the same patentable invention. Hofvander has not satisfied its burden.

Contrary to the views expressed in Hofvander's Main Brief at Final Hearing (HB 1, first para.), we conclude that the invention defined by Hofvander's claims designated as corresponding to the count and the invention defined by Visser's claims designated as corresponding to the count are not directed to obtaining amylose-free starch by suppressing/inhibiting the GBSS gene by use of any effective antisense construct. Rather, we conclude that the invention of Hofvander's claims designated as corresponding to the count and the invention of Visser's claims designated as corresponding to the count are directed to very specific antisense constructs and methods of using very specific antisense constructs to obtain amylose-free starch by suppressing/inhibiting the GBSS gene. None of Hofvander's or Visser's claims designated as corresponding to the count are generally directed to the successful use of antisense technology to suppress/inhibit expression of the GBSS gene in potato plants.

Accordingly, we need not consider whether "Hofvander has clearly shown that the use of fragments and the use of full length sequence both achieve essentially complete suppression/inhibition of the GBSS gene and thus both produce essentially amylose-free starch" (HB 27, first full para.). We conclude that the distinct chemical structures of the fragments defined by, and used in, Hofvander's claims and the full length PGBSS cDNA or genomic DNA sequences defined by, and used in, Visser's claims, render the subject matter Hofvander claims separately patentable from the subject matter Visser claims. Contrary to Hofvander's view (HB 28, last sentence), Hofvander's and Visser's claims do not define the same patentable invention even if antisense constructs comprising Visser's full length GBSS cDNA and genomic DNA sequences and antisense constructs comprising Hofvander's fragments of the PGBSS gene are in fact functionally equivalent for suppressing PGBSS expression.

We cannot disregard the distinct chemical structures of the compounds recited in Hofvander's and Visser's claims. All claim limitations must be considered when determining the patentability of an invention over the prior art. In re Lowry, 32 F.3d 1579, 1582, 32 USPQ2d 1031, 1034 (Fed. Cir. 1994).

Therefore:

We grant Visser's Preliminary Motion No. 1 under 37 CFR § 1.633(b) for judgment that there is no interference in fact

(Visser's Preliminary Motion 1 (Paper No. 17)), because none of Visser's claims designated as corresponding to Count 1 are directed to the same patentable invention as any of Hofvander's claims designated as corresponding to Count 1 (Paper No. 17, p. 2, para. 2) (GRANTED);

We deny Visser's Preliminary Motion No. 2 under 37 CFR § 1.633(a) for judgment that Claims 1, 4, and 6 to 23 of Hofvander's involved application, filed November 24, 1993, designated as corresponding to the count, are unpatentable under 35 U.S.C. § 102 over Hergersberg (VDX 1), and/or under 35 U.S.C. § 103 in view of the combined teachings of Hergersberg and van der Leij (VDX 3) (Paper No. 18) (DENIED);

Visser's Preliminary Motion No. 3 under 37 CFR § 1.633(a) for judgment that Hofvander's Claims 1, 4, 6-20, and 22 are unpatentable under 35 U.S.C. § 112, first paragraph (Paper No. 19) stands dismissed (DISMISSED);

We dismiss Visser's contingent Preliminary Motion 4 under 37 CFR § 1.633(a) for judgment that Hofvander's Claims 1, 4, and 6-23 are unpatentable under 35 U.S.C. § 102 over Visser's 1991 publication (VDX 8) (Visser's Preliminary Motion No. 4 (Paper No. 20)) as contingent on denial of Visser's Preliminary Motion No. 1 (Paper No. 17)) (DISMISSED).

We dismiss Visser's Preliminary Motion No. 5 under 37 CFR § 1.633(c)(4) to have Visser's Claims 1, 4, 8, 11, 13-20, 22, and 24-27 designated as not corresponding to the count (Paper No. 21) (DISMISSED).

We dismiss Visser's contingent Preliminary Motion No. 6 (contingent upon denial of Visser's Preliminary Motions 1-5) under 37 CFR § 1.633(c)(1) to redefine the interfering subject matter by substituting new Count V-1 for Count 1 (Paper No. 22) (DISMISSED).

We dismiss Visser's contingent Preliminary Motion No. 7 under 37 CFR § 1.633(f) to be accorded benefit of the filing dates of Visser's grandparent application, filed December 1, 1993, and Visser's parent application, filed February 14, 1992, for proposed Count V-1 (Paper No. 23) (DISMISSED).

We dismiss as moot Visser's Request To Add Hofvander's Patent[16] To Interference Pursuant To 37 CFR § 1.642 (Paper

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[16] U.S. Patent 5,824,798 (Paper No. 141), assigned to Amylogene HB, Svalov, Sweden, naming Anneli Tallberg, Per Hofvander, Per T. Persson, and Olle Wikstrom as inventors, issued with the following claims:

> 1. A process for producing an amylopectin-type starch comprising:
> obtaining a potato tissue which has been transformed by introducing into the genome of the potato tissue a gene construct comprising a promoter and a fragment of the potato gene which codes for the information of granule-bound starch synthase inserted in the anti-sense direction, wherein said

No. 141). We conclude that no interference-in-fact exists between subject matter claimed in Visser's involved application

---

fragment essentially has a nucleotide sequence which is selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3;

growing the transformed potato tissue to produce a potato plant containing potato tubers;

producing at least one potato from said potato tubers; and

separating starch from said potato, wherein said starch is an amylopectin-type starch which is essentially free of amylose.

2. The process for producing an amylopectin-type starch according to claim 1, wherein said fragment has a nucleotide sequence of SEQ ID No. 1.

3. The process for producing an amylopectin-type starch according to claim 1, wherein said fragment has a nucleotide sequence of SEQ ID No. 2.

4. The process for producing an amylopectin-type starch according to claim 1, wherein said fragment has a nucleotide sequence of SEQ ID No. 3.

5. The process for producing an amylopectin-type starch according to claim 1, wherein said promoter comprises a CAMV 35S promoter.

6. The process for producing an amylopectin-type starch according to claim 1, wherein said promoter comprises a patatin I promoter.

7. The process for producing an amylopectin-type starch according to claim 1, wherein said promoter comprises a GBSS promoter.

8. The process for producing an amylopectin-type starch according to claim 7, wherein said GBSS promoter has the nucleotide sequence of SEQ ID No. 4.

and subject matter claimed in either of Hofvander's involved application or Hofvander's patent (DISMISSED).

3. Motions to suppress evidence

A. Hofvander Motion To Suppress Evidence (Paper No. 123)

Hofvander has moved to suppress paragraph 11 of the Declaration of Peter M. Bruinenberg (VAX 1) because it purportedly contains inadmissible hearsay under Federal Rules of Evidence 601 and 802 (Paper No. 123). For reasons stated herein above, we did not consider paragraph 11 of the Declaration of Peter M. Bruinenberg (VAX 1) in deciding the issues before us at final hearing. Accordingly, so far as it relates to paragraph 11 of the Declaration of Peter M. Bruinenberg (VAX 1), Hofvander's motion to suppress evidence is DISMISSED.

Hofvander's motion to suppress evidence also contains the following arguments (Paper No. 123, para. 6-10):

> 6. Moreover, the Declaration as a whole is not reliable. The First Bruinenberg Declaration is filled with errors and inconsistencies. For example, in Paragraph 8, Dr. Bruinenberg stated that he has allegedly compared the gene sequence in the Hofvander application [SEQ ID No. 5] versus the gene sequence in the Visser application. Dr. Bruinenberg concluded that there were 4,707 matches, 92 mismatches and 762 unmatched base pairs. However, the total of those three numbers is greater than the 4,964 nucleotides in Hofvander SEQ ID No. 5. Moreover, the Visser sequence of Figure 3 is even shorter than the total number of matches that are said to be between the two sequences. . . . .
>
> 7. A further example in the Declaration is where Dr. Bruinenberg stated that the Hergersberg antisense sequence and Sequence ID No. 1 of the Hofvander

> applications start at the same basepair. As admitted by Dr. Bruinenberg during cross examination, that statement is wrong. The Hergersberg 275 basepair fragment in an antisense direction would start with 243 and go backwards, while Sequence ID No. 1 of Hofvander would start at base pair 342 and go backwards. . . . .
>
> 8. Dr. Bruinenberg's statement that the Hergersberg antisense sequence discloses 80% of Hofvander Sequence ID No. 1 was also admitted to be wrong. Further, the statement that the "extra 20 percent of Hofvander sequence ID No. 1 are promoter sequences, i.e., noncoding DNA sequences" was also admitted to be wrong. . . . .
>
> 9. Dr. Bruinenberg incorrectly identified leader sequence in both Hergersberg (pages 28-29)(HX 41) and in SEQ ID No. 1 of Hofvander (HX 40) as being promoter sequence. . . . .
>
> 10. Even Dr. Bruinenberg could not believe the errors in his Declaration . . . .

Visser responds to Hofvander's arguments as follows (Paper No. 134, p. 6):

> [A]lthough Hofvander has only moved to suppress paragraph 11 of the Bruinenberg Declaration, Hofvander opines that the Bruinenberg Declaration as a whole has been shown to be unreliable. The basis for contending that the declaration as a whole is unreliable is set forth in paragraphs 6-10 of Hofvander's statement of material facts. Although Dr. Bruinenberg admittedly made errors in other portions of the Bruinenberg Declaration, this is not a reasonable basis for concluding that the test results set forth in paragraph 11 of the Bruinenberg Declaration are unreliable.

We agree with Visser that Hofvander's motion to suppress evidence is primarily directed to paragraph 11 of the Bruinenberg Declaration. To the extent Hofvander argues that Bruinenberg's Declaration is as whole unreliable, we find that the argument

relates to the weight to be accorded the other evidence therein, not its admissibility.

B. Visser's motions to suppress evidence

(1) Visser has moved to suppress Hofvander Affidavit Exhibit 2, the Declaration of Per Persson (HAX 2), because it purportedly contains inadmissible hearsay under Federal Rules of Evidence 601 and 802 (Paper No. 116). For reasons stated herein above, we did not consider the Declaration of Per Persson (HAX 2) in deciding the issues before us at final hearing. Accordingly, Visser's first motion to suppress evidence (Paper No. 116) is DISMISSED.

(2) Visser has moved to suppress "the portion of the Declaration Pursuant To 37 C.F.R. §1.132 of Per Persson executed October 25, 1994 . . . [(VDX 11)] which was submitted during ex parte prosecution of the Hofvander . . . application relating to the experiments contained in paragraphs 2, 3 and 5[, etc.,]" because it purportedly contains inadmissible hearsay under Federal Rules of Evidence 601 and 802 (Paper No. 117). For reasons stated herein above, we did not consider this evidence in deciding the issues before us at final hearing. Accordingly, Visser's second motion to suppress evidence (Paper No. 117) is DISMISSED.

(3) Visser has moved to suppress "the portion of Hofvander . . . Affidavit Exhibit 3 [(HAX 3)] relating to the

amylose content, viscosity and storage stability experiments" because it purportedly contains inadmissible hearsay under Federal Rules of Evidence 601 and 802 (Paper No. 118). For reasons stated herein above, we did not consider this evidence in deciding the issues before us at final hearing. Accordingly, Visser's third motion to suppress evidence (Paper No. 118) is DISMISSED.

(4) Visser has moved to suppress "the portion of the redirect examination of Lars Rask relating to Hofvander Documentary Exhibit 45 (HR 165, l. 19 - HR 169, l. 14) on the grounds that this portion of the redirect examination is beyond the scope of the cross-examination and . . . comprises inadmissible hearsay" under Federal Rules of Evidence 601, 611 and 802 (Paper No. 119). For reasons stated herein above, we did not consider this evidence in deciding the issues before us at final hearing. Accordingly, Visser's third motion to suppress evidence (Paper No. 118) is DISMISSED.

4. Disposition

It is

ORDERED that, on the record before the Board of Patent Appeals and Interferences, there exists no interference-in-fact between subject matter defined by Claims 1, 4, 6-23 and 50 of Hofvander's U.S. Application 08/070,455 and Claims 1, 4-8, 11, 13-20, and 22-27 of Visser's U.S. Application 08/294,619 because

none of Claims 1, 4, 6-23 and 50 of Hofvander's involved application are drawn to the "same patentable invention" as Claims 1, 4-8, 11, 13-20, and 22-27 of Visser's involved application;

FURTHER ORDERED that, on the record before the Board of Patent Appeals and Interferences, party PER HOFVANDER; PER T. PERSSON; ANNELI TALLBERG, deceased, by LENNART HANSSON, Legal Representative; and OLLE WIKSTROM, is not entitled to a patent containing Claim 6 of Hofvander's U.S. Application 08/070,455, filed November 24, 1993;

FURTHER ORDERED that, on the record before the Board of Patent Appeals and Interferences, party RICHARD G.F. VISSER, EVERT JACOBSEN, and WILLEM J. FEENSTRA, is not entitled to a patent containing Claim 23 of Visser's U.S. Application 08/294,619, filed August 23, 1994;

FURTHER ORDERED that, on the record before the Board of Patent Appeals and Interferences, Visser has not shown that party PER HOFVANDER; PER T. PERSSON; ANNELI TALLBERG, deceased, by LENNART HANSSON, Legal Representative; and OLLE WIKSTROM, is not entitled to a patent containing Claims 1, 4, 7-23 and 50 of Hofvander's U.S. Application 08/070,455, filed November 24, 1993;

FURTHER ORDERED that, on the record before the Board of Patent Appeals and Interferences, Hofvander has not shown party RICHARD G.F. VISSER, EVERT JACOBSEN, and WILLEM J. FEENSTRA, is

not entitled to a patent containing Claims 1, 4-8, 11, 13-20, 22, and 24-27 of Visser's U.S. Application 08/294,619, filed August 23, 1994;

FURTHER ORDERED that the involved applications be remanded to the examiner in charge for further action consistent with this decision; and

FURTHER ORDERED that a copy of this decision be given an appropriate paper number and entered into the file records of Hofvander's U.S. Application 08/070,455, filed November 24, 1993, and Visser's U.S. Application 08/294,619, filed August 23, 1994.

ANDREW H. METZ)
Administrative Patent Judge)
)
)
TEDDY S. GRON) BOARD OF PATENT
Administrative Patent Judge) APPEALS AND
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APPENDIX A

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MOLECULAR CLONING AND PARTIAL CHARACTERIZATION OF THE GENE FOR GRANULE-BOUND STARCH SYNTHASE FROM A WILDTYPE AND AN AMYLOSE-FREE POTATO (*SOLANUM TUBEROSUM* L.)

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The gene encoding granule-bound starch synthase (GBSS), which determines the presence of amylose in reserve starches, has been isolated from a wild-type and from an amylose-free potato by using a potato GBSS cDNA. From the analysis of five genomic GBSS clones, isolated from the wild-type potato, it is shown that GBSS is a single copy gene in potato. GBSS messenger RNA was shown to be present in a number of different tissues, but was most abundantly found in stolons and tubers. Southern blot analysis of the GBSS genes from both potato genotypes revealed that the amylose-free mutant, which lacks GBSS activity and protein, does not contain a large structural lesion in the GBSS gene. The GBSS messenger RNA was even found to be present far more abundant in the mutant than in the wild-type potato.

Key words: granule-bound starch synthase genomic clones; *Solanum tuberosum*; GBSS RNA; amylose-free potato

Introduction

Starch in potato tubers consists of 20–25% amylose and 75–80% amylopectin, which is almost identical to the ratio of amylose/amylopectin in maize endosperm [1]. In maize the *waxy* (Wx) locus, which encodes the (major) granule-bound starch synthase, determines the presence of amylose in endosperm tissue, pollen and the embryosac [2,3]. Many recessive mutations of the Wx locus in maize, resulting in the absence or decrease of the granule-bound starch synthase activity, have been identified and mapped genetically [4].

The availability of these mutants has enabled the identification, isolation, and further analysis of the Wx locus in maize [3,5,6]. In maize, where more than 40 mutant alleles of the Wx locus are available, it has been shown that most of the investigated mutants have no Wx protein [2]. Wessler and Varagona [5] found that 50% of these Wx mutations were the result of a structural lesion (> 50 bp).

In spite of the fact that potato is an important crop and one of the major starch producing plant species there is much less known about the synthesis and breakdown of starch in this species than in maize. However, it is known that, like maize kernels, potato tubers contain a granule-bound starch synthase (GBSS) involved in the production of amylose [7,8].

The isolation and characterization of the potato GBSS protein has been described [9] and recently a mutant (amf-1) of the potato containing amylose-free starch was isolated, lacking GBSS activity and protein in the starch granules [10].

To determine whether the amf-1 mutation is a structural lesion in the GBSS gene we analyzed wildtype and mutant potato with respect to this gene. We have cloned and characterized several genomic GBSS clones from wildtype

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0168-9452/89/$03.50

and amf-1 potato. We also analyzed the presence and/or expression of RNA of the GBSS locus in wildtype and mutant potato.

Materials and Methods

Plant materials

The *Solanum tuberosum* clones H7322 (AM 79.7322, $2n = x = 12$), 86.040 (amylose-free mutant amf-1, $2n = x = 12$, derived after a mutagenic treatment of H7322 leaf strips [10]), 87.1030/14 ($2n = 2x = 24$) and 87.1029/15 (amylose-free F_2 plants $2n = 2x = 24$ [11]) were used as source for RNA and DNA isolation. The plants were grown in a greenhouse at 18 °C/16 °C day/night temperature and 16 h light.

Genomic library construction

DNA was isolated from young H7322 and 86.040 potato leaves and cesium chloride purified according to Schwarz-Sommer et al. [12]. The genomic libraries were constructed essentially as described by Maniatis et al. [13]. Briefly, potato DNA was partially digested with restriction enzyme MboI and size fractionated on a 0.7% agarose gel; fractions containing 14–22 kb fragments were cut out of the gel and isolated by electroelution in 1 × TAE (40 mM Tris acetate, 1 mM EDTA). The electroeluted DNA was applied on a DE52 cellulose (Whatman) column, washed several times with TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.4), eluted with 1 M NaCl, phenol extracted and precipitated with absolute ethanol. Phage lambda EMBL4 DNA [14] was digested with restriction enzymes BamHI and SalI and annealed in the presence of Mg^{2+} as described by Maniatis et al. [13]. The DNA was fractionated onto a 10–40% potassium acetate density gradient [13] and the purified EMBL4 arms were used in the ligation mixture.

EMBL4 DNA was ligated to the electroeluted size fractionated potato DNA, packaged and used to transfect *Escherichia coli* strain K 803 [3]. The genomic libraries were transferred to Nitrocellulose filters (Schleicher and Schüll BA85 0.45 μM) and screened by plaque hybridization [15] to the random primed labelled [16] insert of the granule bound starch synthase cDNA clone pWx 1.3 (3'-end [17]). Hybridizing plaques were purified by two further rounds of plaque hybridization and DNA was prepared form large scale liquid lysates as described in Maniatis et al. [13].

Plant RNA preparation

Total RNA was isolated from various tissues by grinding the frozen material to a fine powder using a Waring blendor. To 1 g of powdered material, 4 ml (60 °C) extraction buffer (0.2 M NaAc, pH 8.5, 1% SDS, 10 mM EDTA) and 2 ml phenol were added. After vigourous vortexing for 2 min, 2 ml chloroform was added. The tube was vortexed vigourously and transferred to a waterbath (60 °C) for 5 min with occasional shaking. After centrifugation at 20 000 × *g* for 30 min the aqueous phase was removed and extracted three times with phenol/chloroform and once with chloroform alone. The aqueous phase was made 2 M with LiCl and the RNA was allowed to precipitate for 4–12 h at 4 °C. The RNA was collected by centrifugation for 10 min at 10 000 × *g*, the resulting pellet washed twice with 70% ethanol and finally dissolved in H_2O. The RNA was again precipitated with ethanol after the addition of 1/10 volume of 3 M NaAc pH 5.0, centrifugated at 10 000 × *g*, washed twice with 70% ethanol and dissolved in 100 μl of H_2O. The E260/E280 were measured and the RNA was stored at −20 °C. Poly A+ RNA was isolated using Hybond™-mAP (messenger affinity paper Amersham International Plc, Amersham, U.K.) according to the manufacturers instructions.

RNA and DNA blot hybridization

Total RNA and poly A+ RNA were denaturated with formaldehyde and formamide and electrophoresed in 1% agarose gels [13]. At the end of the run the gel was soaked in H_2O for 10 min and stained for 3 min in 50 mM NaOH, 10 mM NaCl, 5 μg/ml ethidium bromide. Destaining was for 30 min in 0.1 M Tris–HCl (pH 7.5). The RNA was transferred to Gene Screen Plus

membranes (NEN) with 10 × SSPE (0.1 M NaH_2PO_4, 1.5 M NaCl, 10 mM EDTA) and hybridized with random primed labelled [16] GBSS cDNA. RNA dot blots were performed as described by White and Bancroft [18] using *Schizophyllum commune* RNA as a negative control.

Plant DNA isolation for Southern blots was according to Dellaporta et al. [19]. DNA was digested with the indicated restriction endonucleases, electrophoresed in 0.7% agarose gels using standard procedures [13] and blotted onto Gene Screen Plus membrane according to the manufacturers instructions. Hybridizations of DNA blots were performed essentially as described [20,21]. Hybridization of RNA blots was according to the Gene Screen Plus manual in 1 M NaCl, 10% dextran-sulphate, 1% SDS supplemented with 100 μg/ml salmon sperm DNA for 40 h at 65 °C. Blots were washed three times with 2 × SSC 1% SDS at 65 °C and once with 0.1 × SSC, 1% SDS at room temperature.

The potato GBSS cDNA was isolated from a cDNA library established from poly A⁺RNA from potato tubers using a maize waxy (GBSS) cDNA [6] as probe. Subcloning of the cDNA in plasmid pUC9 yielded plasmids pWx 1.1 (5′-end of the potato GBSS cDNA, and pWx 1.3 (3′-end of the potato cDNA [17]) and pGB6 (pUC 18 with the two EcoRI cDNA fragments from pWx 1.1 and pWx 1.3). These plasmids were isolated as described [22]. The cDNA inserts, to be used as probes, were isolated by EcoRI or BamHI (pGB6) restriction followed by agarose gel electrophoresis and the 'freeze squeeze' method [23].

Probes were labelled by random primed directed synthesis with α^{32}PdCTP to high specific activity according to Feinberg and Vogelstein [16].

Results and Discussion

Molecular analysis of the GBSS genes from the wildtype and amf-1 mutant

Consistent with the presence of a single EcoRI site in the GBSS cDNA sequence, two bands at 4.2 and 4.4 kb, respectively, are evident on Southern blots containing EcoRI digested potato DNA, when probed with either the 5′- or 3′-end of the cDNA (data not shown). In DNA restricted with EcoRI and hybridized to the 2.4-kb insert from pGB6 (containing the total GBSS cDNA) these two bands could also be discerned, whereas HindIII restricted DNA showed three bands with a molecular weight of 2.2, 1.9 and 1.2 kb (Fig. 1).

When total DNA from both wildtype and amf-1 potato were analyzed by Southern hybridization no differences could be found in molecular weights of the hybridizing bands, using a number of different restriction enzymes. Figure 1 shows an example for EcoRI and HindIII restricted DNA.

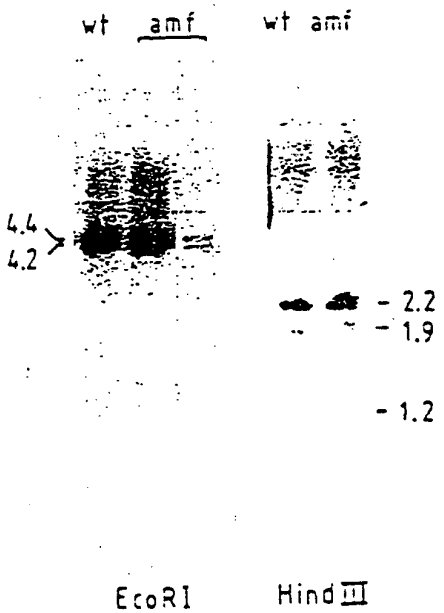

Fig. 1. Autoradiographs of Southern blots of H7822 (wt) and 86.040 (amf) DNA (10 μg/lane) digested with EcoRI and HindIII and probed with the 2.4 kb potato GBSS cDNA. Numbers in the margin indicate molecular weights in kilobase pairs. Only the weaker exposure of EcoRI restricted amf-DNA is shown, a weaker exposure of wt-DNA looks identical.

Molecular cloning of wildtype and amf-1 GBSS genes

The amf-1 mutant does not contain amylose or GBSS protein [10] and, therefore, might carry a structural lesion in the GBSS gene. To investigate this possibility we constructed genomic libraries from the haploid potato clones, H7322 (wildtype) and 86.040 (amf-1 mutant). From these libraries we isolated the GBSS gene for further investigation. The genomic libraries were screened with the 3'-end of the GBSS cDNA (the insert from pWx 1.3).

Screening of 6×10^5 recombinant H7322 phages with random primed labelled probe gave 6 hybridizing plaques. All six clones (denoted LGBSS^{wt}-4, 4-1, 5, 6, 33 and 41) hybridized both to the 5'- and to the 3'-region of the GBSS cDNA. Purified DNA from 5 clones subjected to restriction endonuclease mapping and Southern hybridization using cDNA fragments as probes. In this way we were able to identify two putative full length genomic clones, based on the presence of the three HindIII sites (LGBSS^{wt}-6 and 41) and three partial genomic clones (Fig. 2A). The two full lengthy clones gave bands which comigrate with the bands detected on a genomic Southern blot as shown in Fig. 1 and from these two clones a partial restriction map has been constructed (Fig. 2B). The ends of the transcription unit have not been localized precisely, but no hybridization was observed left from the sec-

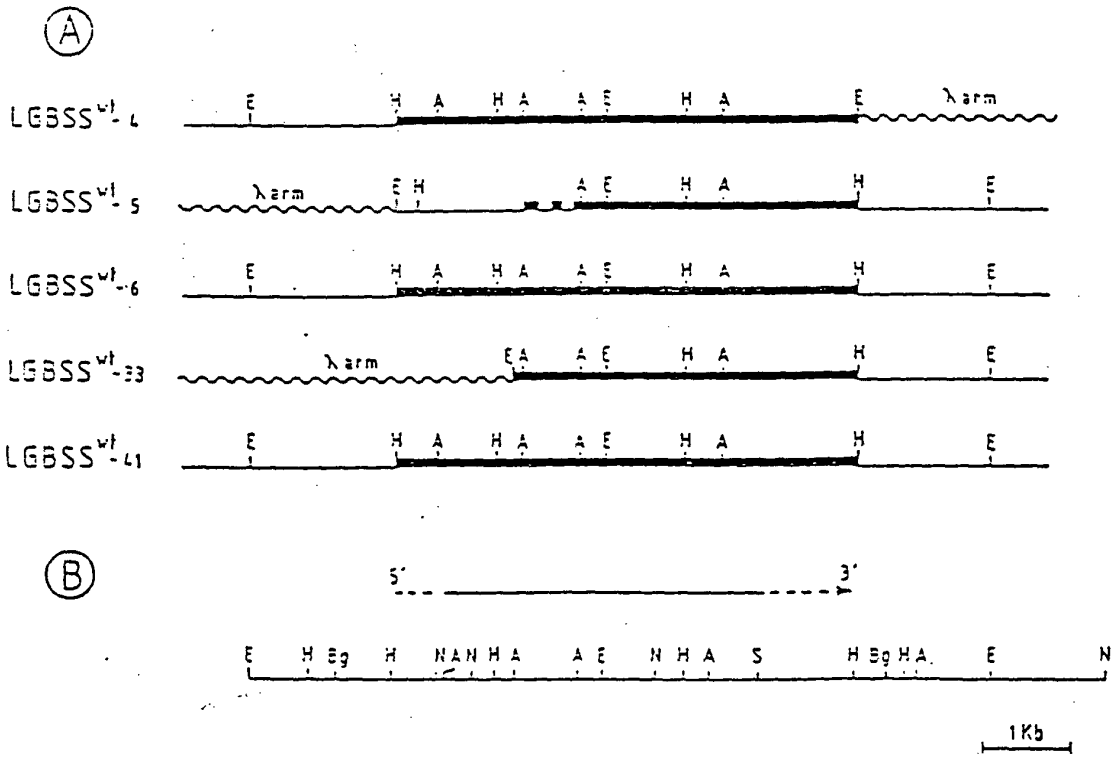

Fig. 2. (A) Restriction endonuclease maps of different H7322 genomic clones. The solid bars indicate the maximum extent of homology (when cut with either EcoRI or HindIII) with the potato GBSS cDNA as determined by Southern blot analysis. The broken solid bar in LGBSS^{wt}-5 indicates that there are still some GBSS sequences in front of the AvaII site. (B) Partial restriction endonuclease site map of the potato GBSS gene. Arrow indicates the direction of transcription, the solid part indicates the part of the gene which shows hybridization with the cDNA. Restriction sites are indicated as letter abbreviations: A, AvaII; Bg, BglII; E, EcoRI; H, HindIII; N, NsiI; S, SpeI.

ond NsiI site or right from the SpeI site when the DNA was probed with the cDNA inserts. All five clones have, for the parts they have in common, similar restriction patterns with the exception of LGBSS^{-}-5 (Fig. 2A). This clone does not contain the 1.2-kb and the 2.2-kb HindIII fragments, but instead displays one larger HindIII fragment of about 2.7 kb. Furthermore, this particular clone lacks the two smaller AvaII fragments and shows instead a larger (~5.0 kb) fragment when probed with the 5'-end from the cDNA. Hybridization of AvaII and HindIII digested DNA from clone LGBSS^{-}-5 with the subcloned 1.2-kb HindIII GBSS 5'-end containing fragment from LGBSS^{-}-41 showed no hybridizing bands, indicating that there is only a small stretch of 5'-region present in this particular clone (as is indicated in Fig. 2A by the boxed region). These different results are most probably a consequence of artifacts due to the cloning procedures. The characterization of the isolated genomic GBSS clones showed that they were all derived from one and the same gene. The fact that all wildtype genomic clones contain, for the hybridizing parts they have in common, restriction sites at exactly the same relative positions towards each other as those in the plant confirms that we isolated the GBSS gene. This is furthermore supported by the presence of the second NsiI site at the 5'-end of the gene and the presence of the unique SpeI site at the 3'-end of the gene, which are present in the GBSS cDNA. As these experiments were performed with DNA from a monohaploid potato clone (H7322) their results support the view that the GBSS locus in potato consists of a single gene.

Screening of 3×10^5 recombinant 86.040 phages gave four clones which were further characterized. Two of the clones which were considered full length clones, because they contained the two EcoRI fragments and the three HindIII fragments as shown in Fig. 1. Also in this case the mutant clones were, for the parts they had in common, identical to each other and to the wildtype clones. We compared the putative full length wildtype clone LGBSS^{-}-6 and mutant clone LGBSS^{amf}-32 by restriction endonuclease mapping with eight different restriction enzymes as well as by double restrictions with HindIII. This yielded hybridizing fragments with a size varying from 200 to 1400 bp, but again no differences were found. Futhermore, analysis of the subcloned HindIII fragments of these two clones with so called 'four cutter' restriction enzymes did not reveal any differences in DNA fragment patterns (data not shown). Therefore, we must conclude that either there is no large structural lesion within the coding sequence of the GBSS gene or, if there is one, that it must be smaller than 50 bp. Deletions or insertions smaller than 50 bp can not be detected by this approach. In the former case there still can be a base substitution. In maize structural lesions of less than 50 bp were found in 9 out of 22 stable *ux* mutations [5].

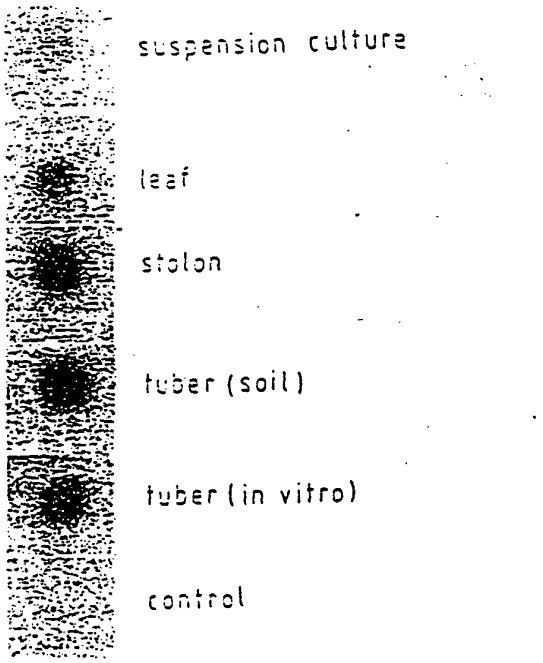

Fig. 3. RNA dot blot analysis of different potato tissues. Total RNA of various tissues of H7322 was spotted onto Gene Screen plus membranes and hybridized to the 2.4 kb GBSS cDNA. RNA amounts were: 8 μg (suspension culture); 8 μg (leaf); 5 μg (stolons); 2 μg (soil grown tuber); 2 μg (minituber); 5 μg (control, *Schizophyllum commune* RNA).

Expression of the GBSS gene

In potato we found that the GBSS gene is expressed in a number of different tissues. Hybridization of RNA from leaves, suspension cultures, stolons, minitubers and soil grown tubers with the cDNA clone was observed (Fig. 3). The messenger is most abundantly expressed in stolons and tubers. The amount of messenger formed in leaves is only 1/10 of the amount found in tubers. The cDNA hybridized to a messenger RNA of about 2.4 kb both in tubers (Fig. 4) and in leaves (data not shown). As the amf-1 mutation is also expressed in different tissues [10], we investigated whether this mutant still produces transcripts of the GBSS gene. For this purpose we isolated RNA from tubers of mutant amf-1. Also in this case the RNA preparation showed a single hybridizing band with a calculated size of 2.4 kb (Fig. 4). A similar hybridizing signal was obtained with poly A^+ RNA from tubers and leaves (not shown). One striking difference between wild-type and mutant GBSS transcript was the amount formed, which was at least a factor three higher in the mutant.

The potato cDNA hybridized to only one transcript of 2.4 kb in both leaves and tubers (Fig. 4 and data not shown). This result is consistent with the expression of only one gene encoding the GBSS as was concluded from the results from the genomic clones. In contrast to the situation in maize where the GBSS (waxy) gene is only expressed in endosperm, pollen and embryosac and thus is tissue specific [2,6], in potato hybridization of the cDNA was observed in all tissues tested (Fig. 3). However, the amount of messenger varied considerably according to the tissue investigated. It was most abundantly present in those organs where reserve starch is formed: tubers and stolons [24,25]. Reserve starch in potato contains a higher amount of amylose than assimilatory starch [26] and so although there does not seem to be a tissue specific expression of the GBSS gene in potato, there seems to be a relationship with the amount of amylose formed.

In maize two *wx* mutants were characterized at the RNA level: wx^{m-6} [3] which forms less messenger of an altered size and wx^{B7} [where no messenger is formed. One possi... hypothesis to explain the presence and abundance of the GBSS transcript in the amf-1 mutant is that the protein regulates the transcription of its gene by feedback inhibition.

The results obtained supply two possible explanations for the nature of the amf-1 mutation: (1) there is an alteration in the GBSS gene, but it is smaller than 50 bp and does not interfere in the transcription of the gene or (2) amf-1 is a mutation in another gene.

Genes other than *Wx*, but showing a similar drastic effect on amylose synthesis have so far not been reported in literature. In rice mutant genes which condition low amylose content of

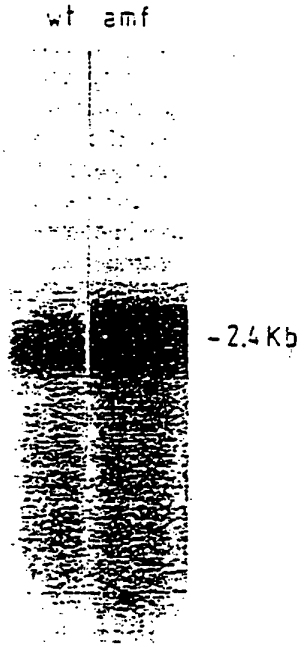

Fig. 4. Northern blot analysis of total tuber RNA isolated from H7322 (wt) and 86.040 (amf) potato. Ten micrograms of each was applied and probed with the 2.4 kb GBSS cDNA. The size of the transcript is indicated in kb and was determined by comparison with RNA standards that are not shown.

starch have been reported [27,28]. These so called *dull* genes for low amylose content are located at 5 loci and segregated independently of the *Wx* locus. They have an additive effect in lowering the amylose content, but not to the point of total absence of amylose [28].

Acknowledgements

We like to thank Dr. W. Rohde for his help in the constructions of the lamda EMBL4 libraries. This work was supported by a grant from ISP (Integraal Structuur Plan Noorden des Lands).

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APPENDIX B

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

VISSER et al. Group Art Unit: 1804

Serial No.: 07/835,886 Examiner: D. FOX

Filed: February 14, 1992

For: POTATO PLANT PRODUCTS ESSENTIALLY AMYLOSE-FREE STARCH

DECLARATION UNDER 37 C.F.R. §1.132

Honorable Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

I, Richard G. F. Visser, is familiar with the subject matter of the above-identified application and declares as follows:

1. That I am employed by the Department of Plant Breeding, Agricultural University, The Netherlands.

2. That the isolation and identification of potato granule-bound starch synthase (PGBSS) promoter was described in Visser et al., Plant Mol. Biol., 17:691-699 as follows.

Materials & Methods

A 0.8 kb fragment of the 5' upstream region of the granule-bound starch synthase (GBSS) genomic clone LGBSS^{wt}-6 (Visser et al., Plant Sci., 64:185-192 (1989)), able to complement the *amf* mutant (van der Leij et al., Theor. Appl. Genet., 82:289-295 (1991)) containing the putative promoter sequences and transcription start, was subcloned in pBIN 101.1 (Jefferson et al., Plant Mol. Biol., 14:995-1006 (1990)) to give a transcriptional

fusion with the β-glucuronidase (GUS) gene from Escherichia coli (Fig. 1). From transient expression studies after electroporation of potato cell suspension protoplasts (van der Steege, pers. comm.) it was known that this fragment was sufficient to drive the expression of the gene. The resulting plasmid pPGB-1 was introduced into *Agrobacterium tumefaciens* strain LBA 4404 using direct transformation of competent *Agrobacterium* cells (Hofgen et al., Nucl. Acid Res., 16:9877 (1988)). The integrity of the plasmid in *A. tumefaciens* was verified according to Holmes and Quigley (Holmes et al., Anal. Biochem., 114:193-201 (1981)), *A. tumefaciens* strain AM8706, containing pBI 121 (a CaMV-GUS construct) was used as a control (Visser et al., Theor. Appl. Genet., 78:594-600 (1989)).

3. That the following experiment was performed to demonstrate the expression of the gene under the control of the promoter.

A series of eleven antisense constructs was made based on GBSS cDNA and genomic sequences, the 35S CaMV promoter and the GBSS promoter (Fig. 2). The construction of pGB50 has been described before (Visser et al., Mol. Gen. Genet., 225:289-26 (1991)). The other constructs are based on pBl121 (Jefferson et al., EMBO J, 6:3901-3907 (1987)) and pPGB-1, which is derived from pBl121 by replacing the 35S CaMV promoter with the GBSS promoter (Visser et al., supra).

For the construction of pKGBA50, pGBA10 and pKGBA10, the (GUS) coding region of pBI121 and pPGB-1 was removed via digestion

with *Sma*I and *Sst*I followed by blunting of the *Sst*I site with T4 DNA polymerase and religation. The resulting vectors pBl121S and pPGB-1S were digested with *Xba*I and *BamH*I. For the construction of pKGBA50 the 2.2kb *BamH*I-*Spe*I fragment from pGB2 (Visser et al., 1991a) was ligated in reversed orientation into digested pPGB-1S. For the construction of pGBA10 and pKGBA10 the 3.0kb *Hin*dIII-*Spe*I fragment containing the complete coding region of the GBSS gene (Visser et al., Theor. Appl. Genet., 78:705-714 (1989)) was subcloned in pUC19 (=SUB10; Fig. 2a). The *BamH*I-*Spe*I fragment of SUB10 was ligated in reversed orientation into digested pBl121S or pPGB-1S, respectively.

The partial genomic antisense constructs pGBA20, pKGBA20, pGBA30 and pKGBA30 are based on *BamH*I and *Sst*I digested pBl121 and pPGB-1. The 1.8kb *Hin*dIII-*Nsi*l fragment of the GBSS gene was subcloned in pMTL23 (Chambers et al., 1988) and isolated as an *Sst*I-*BamH*I fragment (=SUB20; Fig. 2a). This fragment was ligated in reversed orientation into pBl121 (=pGBA20) and pPGB-1 (=pKGBA20). The 1.4kb *Sst*I-*Kpn*I fragment of the GBSS gene was subcloned in pUC19 and isolated as an *Sst*I-*BamH*I fragment (=SUB30; Fig. 2a), which was ligated in reversed orientation into pBl121 (=pGBA30) and pPGB-1 (=pKGBA30). For the construction of pKGBA25 an 1.1kb fragment of the GBSS gene (positions 1552 to 2696; van der Leij et al., Mol. Gen. Genet., 223:240-248 (1991)) was amplified via PCR using two 23-mer primers. Via the Sst-primer (GTTACACTGAGCTCATACTATGC), an

*Sst*I-site is introduced at the 5′ end of the fragment, whereas the Xba-primer (CTGCAAGGCTCTAGACAACTGTT) introduces an *Xba*I-site at the 3′ end of the fragment. The 1.8kb *EcoR*I-*Spe*I fragment of the GBSS gene was used as a template in the PCR reaction. The PCR reaction mixture (100 µl) contained 250 ng of template DNA, 50 mM KCl, 10 mM Tris HCL (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µm dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 1-2 U AmpliTaq (Perkin Elmer Cetus) and was covered with mineral oil. The amplification was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus) in 27 cycles of 1 min. 95°C, 2 min. 52°C and 3 min. at 72°C. PCR products were isolated via the freeze-squeeze method (Tautz and Rentz, Anal. Biochem., 132:14-19 (1983)), restricted with *Sst*I and *Xba*I and ligated in reversed orientation into the *Xba*I-*Sst*I digested pPGB-1. For the construction of pKGBA31 the 0.6kb *Sst*I-*Spe*I fragment of the GBSS gene (=SUB31; Fig. 1a) was directly ligated in reversed orientation into the *Xba*I-*Sst*I digested pPGB-1.

4. That with respect to the promoters used in the above constructs, the presence of GBSS promoter resulted in higher degree of inhibition of GBSS gene expression in tubes of *A. tumefaciens* transformed plants that CaMV 35S promoter. That the results are in agreement with the relative strength of the GBSS promoter compared to CaMV promoter as determined in promoter-GUS experiments, i.e., GBSS expression level is three to ten fold higher than the expression level of CaMV 35S promoter. Note Visser et al., supra.

5. That I declare further that all statements made of

my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 24-11, 1993

Richard G. F. Visser
(Declarant)

Attachments: (a) Figure 1
(b) Figure 2

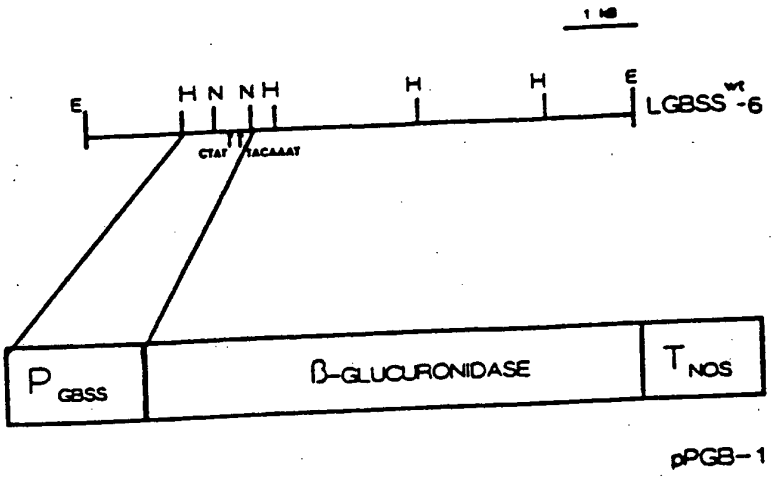

Fig. 1. Schematic representation of the genomic clone LGBSS^{wt}-6 and of the structure of the 5′-GBSS/GUS/NOS-3′ transcriptional fusion of pPGB-1. The 0.8 kb *Hin*d III/*Nsi* I 5′ fragment containing CAAT and TATA boxes was used as GBSS promoter sequence (P_{GBSS}). E, *Eco* RI; H, *Hin*d III; N, *Nsi* I; β-GUS, β-glucuronidase gene; T_{NOS}, nopaline synthase terminator.

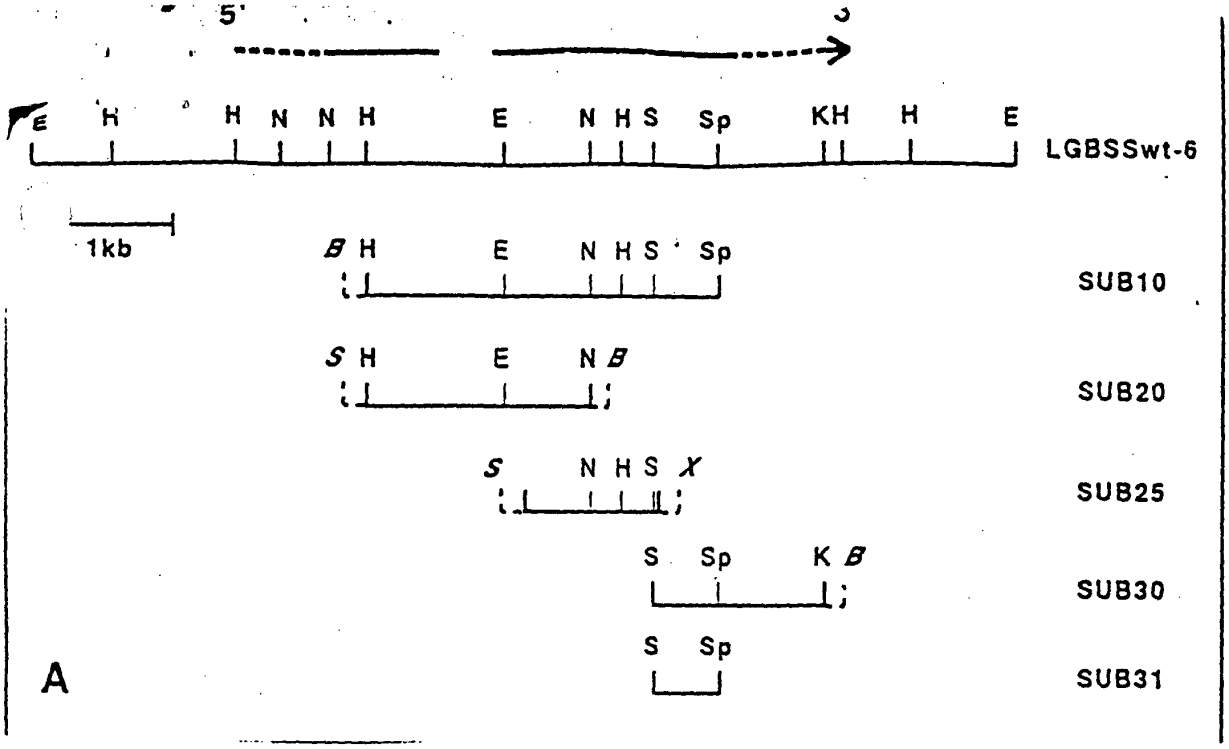

Figure 2: Construction of antisense genes based on the GBSS cDNA and the GBSS gene.
A. Origin of the GBSS gene fragments used for the genomic antisense constructs.
B. Composition of the constructs used in this study.
LGBSS-wt-6: full length genomic GBSS clone (Visser et al, 1989b). The line on top indicates the gene including the promoter region (5' dashed line) and the terminator region (3' dashed line). The arrow indicates the position of the start codon.
B=BamHI; E=EcoRI; H=HindIII; N=NsiI; K=KpnI; Sm=SmaI; S=SstI; Sp=SpeI; X=XbaI (Italics indicate restriction sites in multiple cloning site). RB=right border; LB=left border; NPTII=neomycin phosphotransferase; 35S=35S CaMV promoter; GB=GBSS promoter; T=nopalinesynthase terminator.

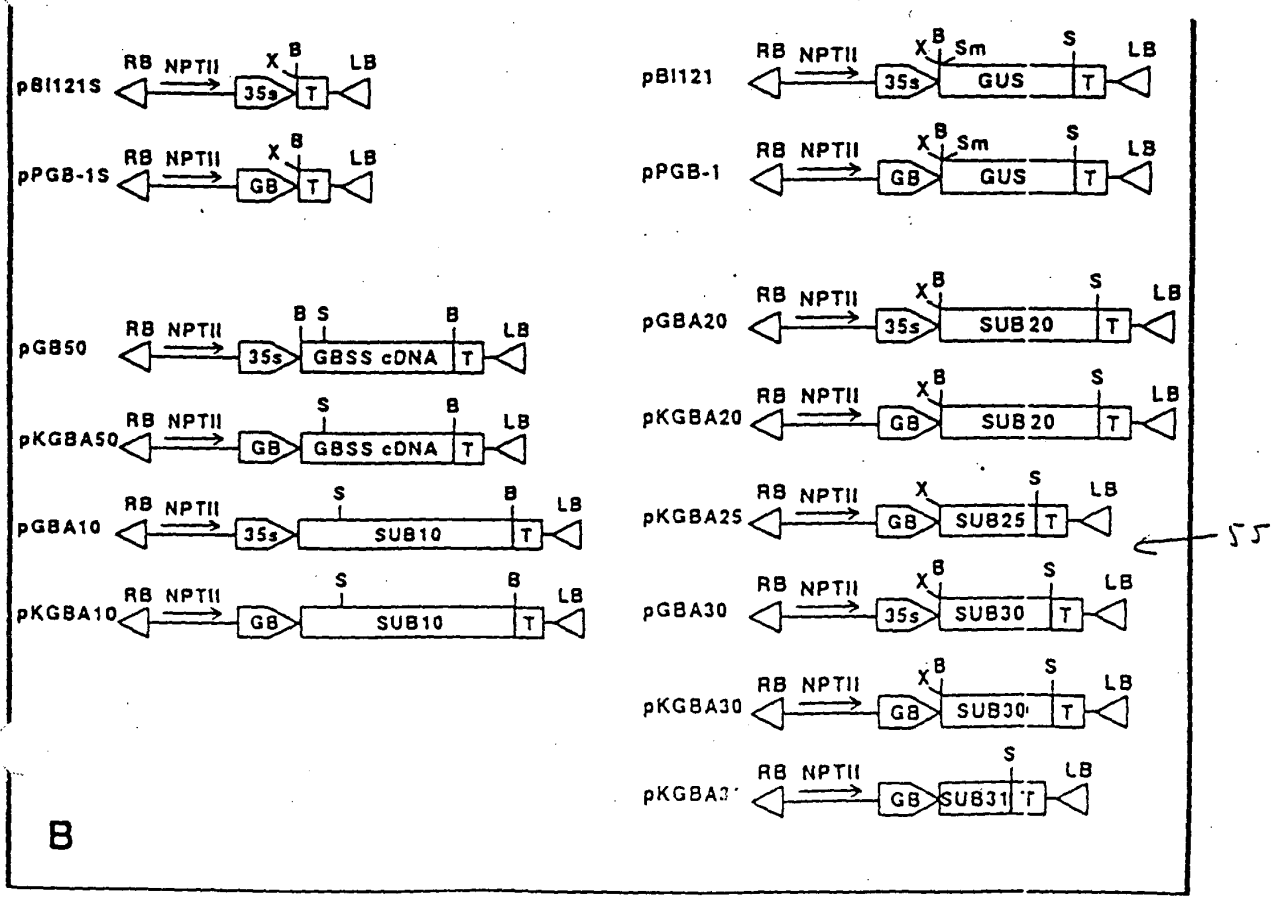

Figure 2

APPENDIX C

Molecular Cell Biology

SECOND EDITION

JAMES DARNELL
Vincent Astor Professor
Rockefeller University

▲

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Professor of Biology, Massachusetts Institute of Technology

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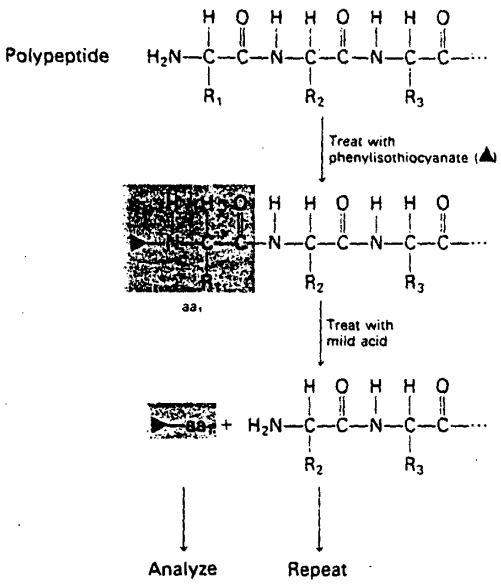


▲ **Figure 6-30** Sequencing a protein by the Edman degradation procedure. The peptide is treated with phenylisothiocyanate, which combines with the amino-terminal residue in the peptide chain, rendering the first peptide bond in the chain labile to treatment with mild acid. The same pair of reactions is carried out repeatedly to remove the amino acids one at a time. After each step, the removed amino acid is chemically identified. In this way, the entire amino acid sequence of a short peptide can be determined.

Table 6-5 Terms used in recombinant DNA research

Genomic DNA	All DNA sequences of an organism
cDNA (complementary DNA)	DNA copied from an mRNA molecule
Plasmid	A small, circular, extrachromosomal DNA molecule capable of reproducing independently in a host cell
Vector	A plasmid or a viral DNA molecule into which either a cDNA sequence or a genomic DNA sequence is inserted
Host cell	A cell (usually a bacterium) in which a vector can be propagated
Genomic clone	A selected host cell with a vector containing a fragment of genomic DNA from a different organism
cDNA clone	A selected host cell with a vector containing a cDNA molecule from another organism
Library	A complete set of genomic clones from an organism or of cDNA clones from one cell type

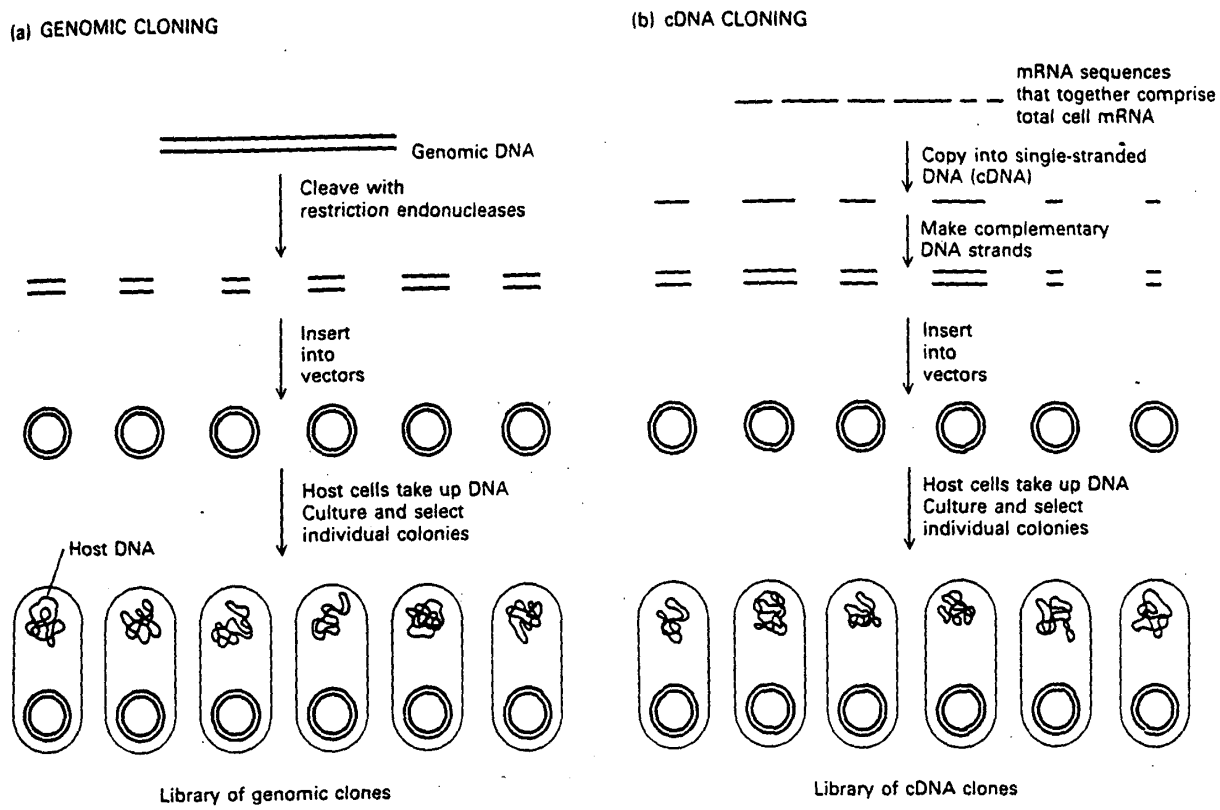
Edman degradation procedure, in which amino acid residues are cleaved from a protein one by one; after each cleavage, the released amino acid is identified (Figure 6-30). Machines called sequenators can perform this reaction on tiny amounts of a pure protein; obtaining an accurate sequence of 50 amino acids is not exceptional.

Recombinant DNA: Selection and Production of Specific DNA

The essence of cell chemistry is to purify sufficient quantities of a particular substance to permit its chemical behavior to be analyzed. Segments of pure samples of identical, relatively short DNA molecules from viruses or plasmids can be isolated directly and subdivided into smaller pieces with the use of restriction endonucleases. But the human genome, for example, contains about 3×10^9 bp, so that cutting roughly at every 3000th base pair would produce a million fragments that could not be separated from each other. This obstacle to obtaining pure DNA samples from large genomes has been overcome by recombinant DNA technology.

Two widely used types of recombinant DNA preparations—genomic clones and cDNA clones—are made. A *genomic clone* contains a fragment of genomic DNA; a *cDNA clone* contains a molecule of *complementary DNA* copied from mRNA by enzymes (Table 6-5). In both, the DNA of interest is linked to a *vector*—most often a bacteriophage or a plasmid that can reproduce independently within a bacterial host. (The most widely used *host-vector systems* are E. coli as host with either a plasmid or bacteriophage λ as the vector.) Recently yeast artificial chromosomes (YACs) have been prepared that can be used as vectors in yeast cells for very large genomic fragments. A *library* consisting of a full set of genomic or cDNA clones can be prepared from the total DNA of an organism or cell type or from the set of cDNA molecules copied from all mRNAs in a cell (Figure 6-31).

The preparation and selection of cDNA and genomic clones are illustrated in the following section by a description of how recombinant DNA containing mouse gl sequences can be obtained.



▲ **Figure 6-31** A comparison of genomic cloning (a) with cDNA cloning (b). In genomic cloning, the genomic DNA must be cleaved with restriction endonucleases before it can be inserted into vectors; in cDNA cloning, the mRNAs must first be copied into double-stranded DNA molecules.

cDNA Clones Are Whole or Partial Copies of mRNA

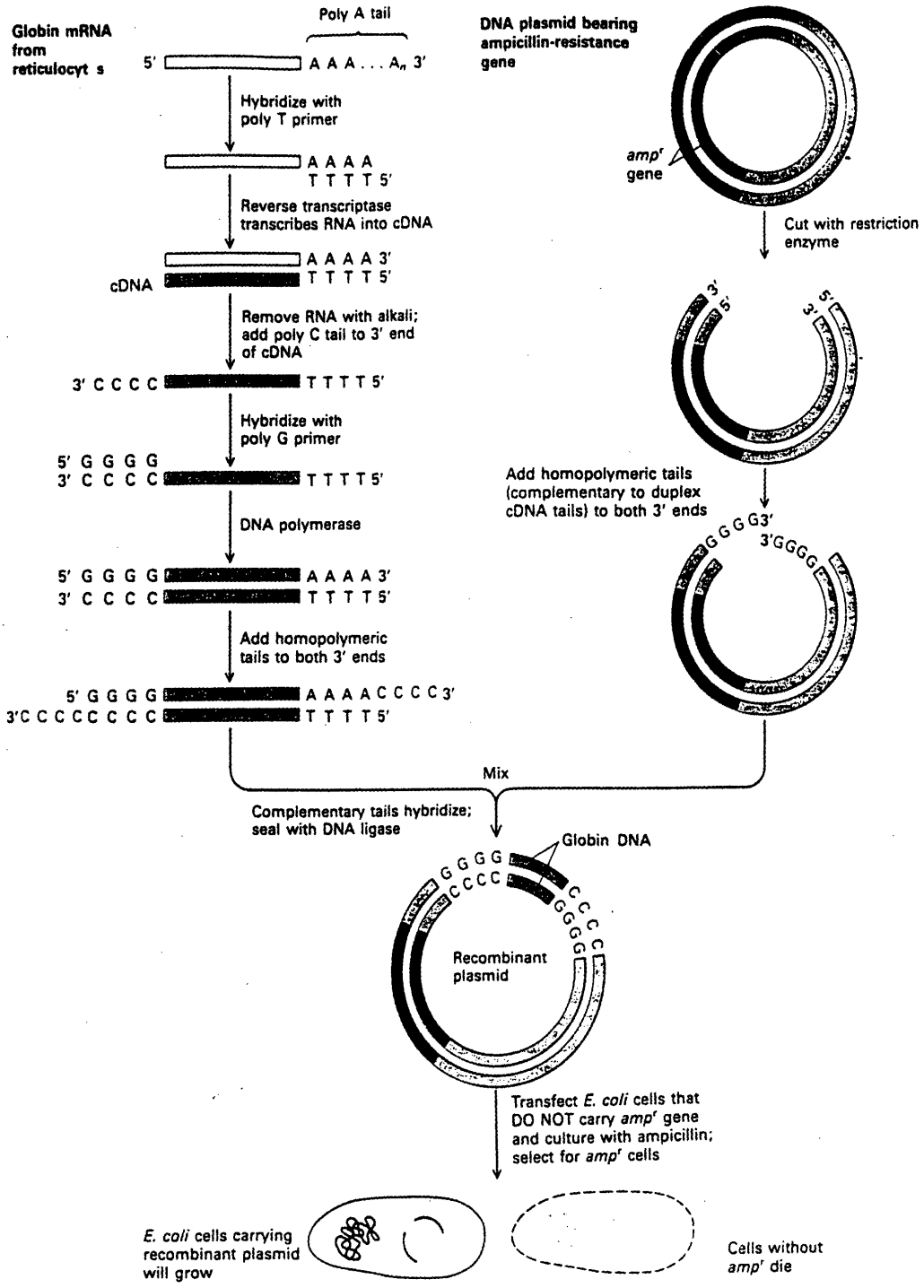
To prepare cDNA clones with globin-encoding sequences (Figure 6-32), the starting material is reticulocytes, erythrocyte (red blood cell) precursors. Over 90 percent of the proteins synthesized by these cells are α- and β-globins and therefore they are rich sources of globin mRNA.

The enzyme *reverse transcriptase* (found in retroviruses; see Figure 5-39) is used to make cDNA clones of the reticulocyte mRNAs. Like the DNA polymerases in cells, this enzyme can build a complementary nucleic acid strand on a template, but only by adding nucleotides to a primer. Thus, before the reverse transcriptase can do its work, a short primer strand must be hybridized to the nucleotides near the 3′ ends of the mRNAs. Fortunately, a single oligonucleotide primer—a string of thymidylate residues (poly T)—serves for most eukaryotic mRNAs, which end in a string of 50–250 adenylate residues (poly A).

After the cDNA copy of the mRNA has been made, the mRNA is removed by an alkali treatment that destroys RNA but does not affect DNA and a duplex DNA is made from the cDNA strand. In one technique, the 3′ end of each cDNA strand is elongated by adding several residues of a single nucleotide (say, poly C) through the action of a *terminal transferase*, an enzyme that adds bases at free 3′ ends. A poly G primer is hybridized with the terminal poly C and this G primer is then elongated by a DNA polymerase. What results is a complete double-stranded DNA copy of the original mRNA.

The next step is to insert the now double-stranded DNA into a plasmid. Plasmids, which occur naturally in almost all bacteria, were originally detected by their ability to transfer genes between bacteria (Chapter 5). It has been shown that a specific region of the plasmid circle, the *replication origin*, must be present to assure replication of the plasmid in a host bacterium.

The plasmid DNA is cleaved once with a restriction enzyme at a point that leaves the replication origin undisturbed. The double-stranded copy of the mRNA-globin is then inserted at the cut site and the circle is rejoined. The



▲ **Figure 6-32** Preparation of a cDNA clone with globin encoding sequences.

first technique, still widely used, for carrying out this insertion is called *homopolymeric tailing*. A homopolymer (say, poly C) is added to the two 3′ ends of the double-stranded cDNA-globin, and a complementary homopolymer (poly G) is added to the 3′ ends of the cut plasmid. When the "tailed" plasmid and DNA-globin are mixed, their complementary single-stranded tails spontaneously hybridize; the resulting circular recombinant molecule can be resealed with the enzyme DNA ligase (Chapters 3 and 12). Specially treated *E. coli* cells take up the plasmid, and the recombinant molecule multiplies along with the cells.

If the chosen plasmid contains a gene for resistance to an antibiotic, the cells that take up the plasmid will grow and multiply in the presence of the antibiotic but the other cells will not. If, at the outset, the number of plasmids allowed to infect the *E. coli* cells is one-tenth or less of the total number of *E. coli* cells, it is very unlikely that more than one plasmid will end up in a recipient cell. As a rule, then, the recombinant DNA in all cells of a colony grown from a single cell will have descended from a single recombinant DNA molecule. In the case described here, 90 percent of the recombinant plasmids would encode α- or β-globin. Because mRNA molecules are often not completely copied, partial sequences also may be cloned. To verify exactly what the plasmid vector contains, the recombinant molecule can be sequenced.

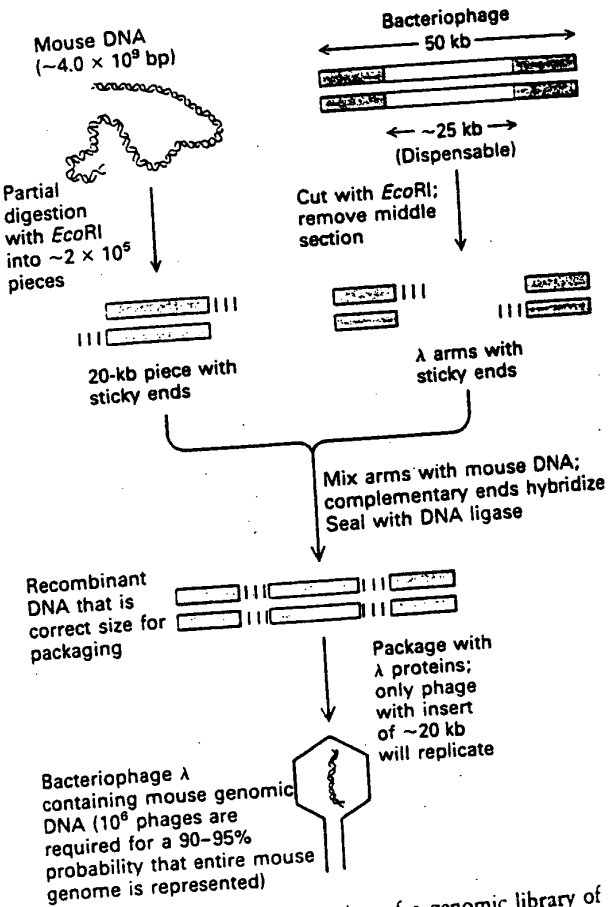
Complementary DNA clones can be prepared from the unpurified mRNA from any cell type, but this produces a random mixture of individual recombinant clones that must be screened to isolate specific ones (see Figure 6-34). It is also possible to use an antibody that reacts with a protein to detect whether an *E. coli* colony (or a plaque if the vector is a bacteriophage) contain the protein encoded by the cloned cDNA.

Genomic Clones Are Copies of DNA from Chromosomes

The most common procedure for preparing and selecting specific clones from genomic DNA—for example, the total collection of DNA in mouse chromosomes—makes use of λ bacteriophage. The DNA of the phage is about 50 kb long, but a center section about 25 kb long can be removed and replaced with foreign DNA without impairing the ability of the phage to infect and reproduce in most *E. coli* cells. A genomic library is a collection of recombinant molecules, maintained either in phage particles or in plasmids growing in bacteria, that includes *all* DNA sequences of a given species. Once it is prepared, the library can be screened for the phage or plasmid that contains the DNA sequence of interest.

The size of a library depends on the amount of DNA in the organism's haploid genome. For example, the human and mouse genomes are between 3 and 4×10^9 bp long. If one of these genomes were divided into fragments about 20 kb long for insertion into bacteriophage λ, then 2×10^5 different recombinant bacteriophage λ particles would be required to constitute a complete library. Because the pieces of DNA are incorporated into phages by chance, about 10^6 recombinant phages are necessary to assure that each DNA piece has a 90–95 percent chance of being included.

The first step in preparing a genomic library is to extract all the organism's DNA from some cell types (Figure 6-33). Sperm cells or early embryos are often used. The DNA is then broken into fragments by a restriction endonuclease, such as *Eco*RI, which cleaves the DNA in a way that produces short, single-stranded, "sticky" ends (AATT) on every fragment (see Figure 6-20). Digestion is stopped when the average size of a fragment is approxi-



▲ **Figure 6-33** The construction of a genomic library of mouse DNA in bacteriophage λ. The total DNA from mouse cells (both sperm cells and embryonic tissue cells presumably have a complete set of sequences) is often used. A single region of the mouse genome, such as the one that encodes β-globin, would occur approximately once in 10^5 particles.

PATENT

Attorney's Docket No. 003300-293

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)
	)
PER HOFVANDER, PER T. PERSSON	)
ANNELI TALLBERG (Deceased),	) Legal Affairs Branch of the
OLLE WIKSTRÖM	) Office of Administrator of
	) Legal Affairs
Serial No. 08/070,455	)
	) Attention: Leonard E. Smith
Filed: June 9, 1993	)
	) [This Application now is pending
For: GENETICALLY ENGINEERED	) in Group Art Unit 1804].
MODIFICATION OF POTATO TO	)
FORM AMYLOPECTIN-TYPE	)
STARCH	)

DECLARATION OF MARIA FALK

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, MARIA FALK, declare:

1. I am an attorney/jurist who is authorized to practice law in Sweden, am affiliated with AWAPATENT AB, Bellevuevägen 46, Box 5117, S-200 71 Malmö, Sweden, and am familiar with the Swedish law with respect to the identification of the heirs of a decedent.

2. I am familiar with the above-identified United States Patent Application Serial No. 08/070,455, the previous Submission Pursuant to 37 C.F.R. § 1.42 and 1.44 that was filed on November 24, 1993, the attachments thereto, and the Decision on Petition Under 37 C.F.R. 1.42 and 1.44 dated November 22, 1994.

3. The main rules in Swedish law regarding the right to inherit are the following.

The first inheritor of a deceased person is the person's descendant (direct heir). However, if the deceased person was

married, the estate will be the property of the surviving spouse. As regards co-habitants, the surviving co-habitant has the right to inherit only the co-habitants joint domicile and household goods.

4. When Anneli Tallberg died on July 11, 1992 she had two children, namely Maria Torper and Olof Torper as evidenced by information contained in the previously submitted Estate Inventory, and she was not married but was a co-habitant with Eskil Persson, identfied in the previously submitted Estate Inventory.

5. The co-habitant Eskil Persson has not requested estate distribution, he is not a residuary legatee, he is under Swedish law not a party of the estate of the deceased Anneli Tallberg in any manner, and is not considered to be one of the heirs of Anneli Tallberg. The only and sole heirs of the estate of Anneli Tallberg are her children Maria Torper and Olof Torper (the latter represented by Tommy Tallberg, Guardian and Legal Representative as previously documented).

6. Under Swedish law, it is permissible and proper for the sole heirs of Anneli Tallberg (i.e. Maria Torper and Olof Torper by his Guardian, Tommy Tallberg) to sign the Declaration of U.S. Patent Application Serial No. 08/070,455 on behalf of the named deceased inventor.

7. I have inspected the entire Estate Inventory that was submitted on November 24, 1993, and have determined that any pages that were previously not translated into English and submitted to the U.S. Patent and Trademark Office are irrelevant to the issue of the authority of the sole heirs (e.g. they identify bank accounts, a Volvo 360 GLE automobile, etc. of the deceased and should be of no interest to personnel of the U.S. Patent and Trademark Office).

8. An authenticated copy of the will of Anneli Tallberg is attached hereto as Exhibit A, and an English translation of this previously was submitted as a part of Exhibit D on November 24,

1993. This document was requested in the November 22, 1994 Decision on Petition Under 37 C.F.R. § 1.42 and 1.44.

9. In summary, under Swedish law, the sole heirs of Anneli Tallberg are her children, Maria Torper and Olof Torper, and the Declaration filed in U.S. Patent Application No. 08/070,455 on November 24, 1993 on behalf of the deceased inventor, Anneli Tallberg is belived to be proper in all respects under the law of Sweden.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the Application or any patent issued thereon.

Date: JANUARY 11, 1995

Maria Falk

Maria Falk

PATENT
Attorney's Docket No. 003300-293

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
)
PER HOFVANDER, PER T. PERSSON)
ANNELI TALLBERG (Deceased),) Office of the Assistant
OLLE WIKSTRÖM) Commissioner for Patents
) Attention: Mr. Vince Turner
Serial No.: 08/070,455)
)
Filed: June 9, 1993)
)
For: GENETICALLY ENGINEERED)
MODIFICATION OF POTATO TO)
FORM AMYLOPECTIN-TYPE)
STARCH)

SUBMISSION PURSUANT TO 37 C.F.R. §1.42 AND 1.44

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

This Application is pending in Group Art Unit 1804, and is based upon International Application No. SE91/00892 filed December 20, 1991. An official Filing Receipt has been issued that identifies LENNART HANSSON as the Legal Representative for the deceased inventor, ANNELI TALLBERG.

The present documentation is being submitted in order to fully satisfy the provisions of 37 C.F.R. §1.42 and 1.44 following a November 23, 1993 discussion with Magdelen Y.C. Greenlief of the U.S. Patent and Trademark Office.

As previously indicated, the joint-inventor, ANNELI TALLBERG, is deceased. This deceased joint-inventor had two children who were her sole heirs. They are Maria Torper (a surviving daughter who is of age) and Olof Torper (a surviving son who is underage). Tommy Tallberg was appointed as Guardian for the surviving underage son, Olof Torper.

Attached as Exhibit A is new Declaration signed by the three living joint-inventors, Maria Torper as heir, and Tommy Tallberg as Guardian for the other heir, Olof Torper.

Attached as Exhibit B is a certified copy of the Estate Inventory of the deceased joint inventor, Anneli Tallberg. This was certified by a Notary Public and the Lund District Court

of Sweden, and is authenticated by Justice B. Stevens, Consul of the United States of America. The sole heirs of the deceased are identified.

Attached as Exhibit C is a certified copy from the Guardianship Book of the Lund District Court with respect to the underage son, Olof Torper, of the deceased joint-inventor, Anneli Tallberg. This was certified by a Notary Public and by the Lund District Court of Sweden, and is authenticated by Justice B. Stevens, Consul of the United States of America. Tommy Tallberg was appointed to serve as Guardian for the underage son, Olof Torper.

Attached as Exhibit D is a verified English translation of the Estate Inventory (i.e., Exhibit B) and the Guardianship Book (i.e., Exhibit C) prepared by Charlotta Kristensson. It will be noted from the Estate Inventory that the deceased, Anneli Tallberg left two children who were the sole heirs (i.e., Maria Torper and Olof Torper). It will be noted from the Guardianship Book that Tommy Tallberg was appointed Guardian or Legal Representative for the underage son, Olof Torper, of the deceased joint-inventor, Anneli Tallberg.

The issuance of a new Official Filing Receipt that reflects the information contained herein respectfully is requested. Documentation in full compliance with 37 C.F.R. §1.42 and 1.44 has been provided.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS

By Benton S. Duffett, Jr.

Benton S. Duffett, Jr.
Registration No. 22,030

Burns, Doane, Swecker & Mathis
George Mason Building
Washington & Prince Streets
P.O. Box 1404
Alexandria, Virginia 22313-1404

Filed: November 24, 1993

GENERAL AUTHENTICATION CERTIFICATE

KINGDOM OF SWEDEN)
CITY OF STOCKHOLM) SS
EMBASSY OF THE)
UNITED STATES OF AMERICA)

I do hereby certify that the official named below, whose true signature and official seal are, respectively, subscribed and affixed to the annexed document, was, on this day, empowered to act in the official capacity designated in the annexed document, to which faith and credit are due.

Örjan Landelius, Counsellor, Second Division, Legal Departme
(Typed Name of Official) Ministry for Foreign Affai
Stockholm, Sweden

(Signature of Consular Officer)

Justice B. Stevens
Consul of the United States
of America
(Name of Consular Officer)

August 23, 1993
(Date)

(SEAL)

DOCKETED
filed 1/19/95

MHH

ADVOKATFIRMAN
HEDENER & PENSER
ADVOKAT LENNART HANSSON AB
ESLÖV

I samarbete med
ADVOKAT WILH. PENSER
ADVOKAT BO GÖRAN PERSSON
ADVOKAT JONAS EILERT

TESTAMENTE

Jag, Anneli Maria Torper-Tallberg, född 15 september 1945, förordnar härmed såsom min yttersta vilja och testamente följande.

1. Volvo NTB 873 till Ulf Torper, 450727 - -
2. 2st rutiga soffor till Eskil Persson, 300416-4152 ✓
3. 2st. Lundquist-bokhyllor till Eskil Persson, ✓ 300416-4152.
4. 1st Denon CD-spelare till Eskil Persson, ✓ 300416-4152
5. 1st Tibetmatta, draknotiv till Maria Torper 740223-3923
6. 1st doktorsring till Olof Torper, 760128-4032-

Lund den 25/6 1992

Anneli Tallberg Torper

Anneli Torper-Tallberg

Att Anneli Torper-Tallberg, som vi personligen känner, denna dag

DOCKETED
filed 11/19/95

vid sunt och fullt förstånd och av fri vilja i bådas vår samtidiga närvaro förklarat ovanstående förordnande utgöra hennes yttersta vilja och därunder tecknat sitt namn, intygar vi särskilt anmodade vittnen.

Lund som ovan

Namn: Bo Persson
Yrke: Leg Läk
Adress: Vegagatan 5
224 71 LUND

Namn: GEORGES GUEDJ
Yrke: SJUKSKÖTARE
Adress: Kollegievägen 134
224 73 LUND

FOTOSTATISK
från
Advokatfirma
Hedener & I
ESLÖV

LUNDS TINGSRÄTT
☐ Avskriften ☒ Kopian
överensstämmer med ~~originalet~~
kopian av originalet

tingsnotarie Lars Persson

Härmed intygas att Lars Persson egenhändigt vidimerat denna handling och att han därtill är behörig.
Lund den 12 augusti 1993
Ex officio:

Helén Kinnman
Notarius Publicus

Dnr 303/93
Avgift SEK 100

NOTARIUS PUBLICUS I LUND

No. 9719

The Ministry for Foreign Affairs in Stockholm hereby certifies that

Mr Lars Persson
District Court
Lund

has issued and signed the foregoing attestation in his official capacity

Fee Stockholm this 18th day of August
Kr U.A. 19 93

Örjan Landelius

EXPEDITIONSAVGIFT
18. 3. 93 KR ÖRE 000:-
UTRIKESDEPARTEMENTET

NOTARIUS

PATENT
Attorney's Docket No. 003300-293

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of PER HOFVANDER, PER T. PERSSON ANNELI TALLBERG (Deceased), OLLE WIKSTRÖM Serial No. 08/070,455 Filed: June 9, 1993 For: GENETICALLY ENGINEERED MODIFICATION OF POTATO TO FORM AMYLOPECTIN-TYPE STARCH	Legal Affairs Branch of the Office of Administrator of Legal Affairs Attention: Leonard E. Smith [This Application now is pending in Group Art Unit 1804].

DECLARATION OF BO BENGTSSON

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, BO BENGTSSON, declare:

1. I am an attorney/jurist who is authorized to practice law in Sweden, am affiliated with AWAPATENT AB, Bellevuevägen 46, Box 5117, S-200 71 Malmö, Sweden, and am familiar with the Swedish law with respect to the identification of the heirs of a decedent.

2. I am familiar with the above-identified United States Patent Application Serial No. 08/070,455, the previous Submission Pursuant to 37 C.F.R. § 1.42 and 1.44 that was filed on November 24, 1993, the attachments thereto, and the Decision on Petition Under 37 C.F.R. 1.42 and 1.44 dated November 22, 1994.

3. When Anneli Tallberg died on July 11, 1992, under Swedish law her sole heirs were her children, Maria Torper and Olof Torper, as evidenced by information contained in the previously submitted Estate Inventory.

4. Under Swedish law the co-habitant, Eskil Persson, identified in the previously submitted Estate Inventory, is not considered to be one of the heirs of Anneli Tallberg, and does not qualify as her heir in any respect.

5. Tommy Tallberg under Swedish law was duly appointed Guardian or Legal Representative for the underage son, Olof Torper, as previously documented.

6. Under Swedish law, it is permissible and proper for the sole heirs of Anneli Tallberg (i.e., Maria Torper and Olof Torper by his Guardian, Tommy Tallberg) to sign the Declaration of U.S. Patent Application Serial No. 08/070,455 on behalf of the named deceased inventor.

7. I have inspected the entire Estate Inventory that was submitted on November 24, 1993, and have determined that any pages that were previously not translated into English and submitted to the U.S. Patent and Trademark Office are irrelevant to the issue of the authority of the sole heirs (e.g., they identify bank accounts, a Volvo 360 GLE automobile, etc. of the deceased and should be of no interest to personnel of the U.S. Patent and Trademark Office).

8. An authenticated copy of the will of Anneli Tallberg is attached hereto as Exhibit A, and an English translation of this previously was submitted as a part of Exhibit D on November 24, 1993. This document was requested in the November 22, 1994 Decision on Petition Under 37 C.F.R. § 1.42 and 1.44. However, this document is not relevant in any way with respect to an identification of the sole heirs of Anneli Tallberg.

9. In summary, under Swedish law, the sole heirs of Anneli Tallberg are her children, Maria Torper and Olof Torper, and the Declaration filed in U.S. Patent Application No. 08/070,455 on November 24, 1993 on behalf of the deceased inventor, Anneli Tallberg is believed to be proper in all respect under the law of Sweden.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the Application or any patent issued thereon.

Date: X

X

Bo Bengtsson

GENERAL AUTHENTICATION CERTIFICATE

KINGDOM OF SWEDEN)
CITY OF STOCKHOLM) SS
EMBASSY OF THE)
UNITED STATES OF AMERICA)

I do hereby certify that the official named below, whose true signature and official seal are, respectively, subscribed and affixed to the annexed document, was, on this day, empowered to act in the official capacity designated in the annexed document, to which faith and credit are due.

Örjan Landelius, Counsellor, Second Division, Legal Departm
(Typed Name of Official) Ministry for Foreign Affa
Stockholm, Sweden

(Signature of Consular Officer)

Justice B. Stevens
Consul of the United States of America
(Name of Consular Officer)

August 23, 1993
(Date)

(SEAL)

ADVOKATFIRMAN
HEDENER & PENSER
ADVOKAT LENNART HANSSON AB
ESLÖV

i samarbete med
ADVOKAT WILH. PENSER
ADVOKAT BO GÖRAN PERSSON
ADVOKAT JONAS EILERT

T E S T A M E N T E

Jag, Anneli Maria Torper-Tallberg, född 15 september 1945, förordnar härmed såsom min yttersta vilja och testamente följande.

1. Volvo NTB 873 till Ulf Torper, 450727 - -
2. 2st rutiga sofför till Eskil Persson, 300416-4152 ✓
3. 2st. Lundquist-bokhyllor till Eskil Persson, ✓ 300416-4152.
4. 1st Denon CD-spelare till Eskil Persson, ✓ 300416-4152
5. 1st Tibetmatta, draknotiv till Maria - Torper 740223-3923
6. 1st doktorsring till Olof Torper, 760128-4032-

Lund den 25/6 1992

Anneli Tallberg Torper

Anneli Torper-Tallberg

Att Anneli Torper-Tallberg, som vi personligen känner, denna dag

vid sunt och fullt förstånd och av fri vilja i bådas vår samtidiga närvaro förklarat ovanstående förordnande utgöra hennes yttersta vilja och därunder tecknat sitt namn, intygar vi särskilt anmodade vittnen.

Lund som ovan

Namn: Bo Persson
Yrke: Leg Läk
Adress: Vegagatan 5
224 67 LUND

Namn: Georges Guedj
Yrke: Sjuksköterare
Adress: Kollegiev. 134
224 73 LUND

FOTOSTATISK
från
Advokatfirma
Hedener & I
ESLÖV

Britt Sahlin Olsson
A Lantz

LUNDS TINGSRÄTT
☐ Avskriften Å Kopian
överensstämmer med ~~min avskrift~~
kopian av originalet
Lars Persson
tingsnotarie Lars Persson

Härmed intygas att Lars Persson egenhändigt vidimerat denna handling och att han därtill är behörig.
Lund den 12 augusti 1993
Ex officio:

Dnr 303/93
Avgift SEK 100

Helén Kinnman
Notarius Publicus

NOTARIUS PUBLICUS I LUND

GENERAL AUTHENTICATION CERTIFICATE

KINGDOM OF SWEDEN)
CITY OF STOCKHOLM) SS
EMBASSY OF THE)
UNITED STATES OF AMERICA)

I do hereby certify that the official named below, whose true signature and official seal are, respectively, subscribed and affixed to the annexed document, was, on this day, empowered to act in the official capacity designated in the annexed document, to which faith and credit are due.

Örjan Landelius, Counsellor, Second Division, Legal Department,
(Typed Name of Official) Ministry for Foreign Affairs
Stockholm, Sweden

(Signature of Consular Officer)

Justice B. Stevens
Consul of the United States
of America
(Name of Consular Officer)

August 23, 1993
(Date)

(SEAL)

DOCKETED
filed 11/24/93

Postadress	Gatuadress	Telefon	Org.-nr	Telefax	Postgiro	Bankgiro
B x 201 241 23 Eslöv	Södergatan 2 241 30 Eslöv	Växel 0413-132 40	12-556209-1255	0413-606 19	634 65 05-8	5362-0928

MHF

ADVOKATFIRMAN
HEDENER & PENSER
ADVOKAT LENNART HANSSON AB
ESLÖV

I samarbete m d
ADVOKAT WILH. PENSER
ADVOKAT BO GÖRAN PERSSON
ADVOKAT JONAS EILERT

19 29 179 23 2 93

B O U P P T E C K N I N G

Med början den 13 augusti 1992 har förrättats bouppteckning efter Anneli Marie Tallberg Torper, Drapavägen 69, 224 74 Lund, som var född den 15 september 1945 och avled den 11 juli 1992.

SK-93

-1508

Den avlidna efterlämnar som dödsbodelägare:

BARNEN

1. Maria Alice Torper, 740223-3923, Drapavägen 69, 224 74 LUND
2. Anders Olof Torper, 760128-4032, Drapavägen 69, 224 74 Lund

Det antecknades att vid förrättningen var närvarande sambon Eskil Persson, Drapavägen 69, 224 74 Lund, numera Kastanjegatan 21:7, 224 56 Lund, testamentstagare Ulf Torper, Sångarevägen 20 E, 224 71 Lund, såsom förmyndare för Olof Torper samt Tommy Tallberg, 401112-4510, Nyckelkroken 25, 226 47 Lund, såsom föreslagen god man för Olof Torper.

Det antecknades att den avlidna efterlämnar testamente av den 25 juni 1992, Bil. 1.

Det antecknas vidare att Eskil Persson påkallat bodelning i anledning av samboförhållandet, Bil. 2.

Boet uppgavs av Eskil Persson. Tillgångar och skulder antecknades och värderas på följande sätt.

TILLGÅNGAR

Bankmedel	
Sparbanken Skåne, Bil. 3	7.379:34
Fordringar	
Svalöf AB, innestående lön	12.157:10
Försäkringskassan, sjukpenning	3.277:--
Överskjutande skatt enligt 1992 års taxering, Bil. 4	12.542:--
Personbil	
Volvo 360 GLE 1988 års modell, 7.600 mil	35.000:--
Insats- och medlemskonto	
Konsumentföreningen Solidar, Bil. 5	476:70
Lösöre, Bil. 6	3.100:--
Fastighet	
Lund Erik Plogpenning 29, Bil. 7, 1/2	492.000:--
Kronor	565.932:14

SKULDER

Länsbostadsnämnden, Bil. 8, 1/2	106.647:41
SPINTAB, Bil. 9, 1/2	299.966:50
Svenska Handelsbanken, Bil. 10	4.295:--
Lunds Energiverk	1.148:--
Malmöhus läns landsting, vårdkostnader	2.800:--
Kronor	414.856:41

AVGÅENDE POSTER

Begravningskostnader		13.908:--
Gravsten		8.500:--
Bouppteckningskostnader		4.500:--
	Kronor	26.908:--

SAMMANSTÄLLNING

Tillgångar		565.932:14
Skulder	414.856:41	
Avgående poster	26.908:--	441.764:41
Behållning i boet kronor		124.167:73

FÖRSÄKRINGAR 212.668:-

TryggHansa/SPP, Bil. 11
Förenade Liv, Bil. 12
Förenade Liv, Bil. 13
SparLiv, Bil. 14
Folksam, Bil. 15
Wasa, Bil. 16

Jag försäkrar härmed på heder och samvete att mina uppgifter till bouppteckningen är i allo riktiga och att inte något med vilja och vetskap är utelämnat. Jag försäkrar vidare att något sådant förvärv som avses i 19 § lag (1941:416) om arvsskatt och gåvoskatt inte förekommit.

Lund den 27/10 1992

Eskil Persson

Vi förrättningsmän intygar att allt blivit rätteligen antecknat och att tillgångarna värderats efter bästa förstånd.

Lennart Hansson
Advokat
Box 201
241 23 Eslöv

Britt Sahlén Olfosson
Sekreterare
Box 201
241 23 Eslöv

Närvarande

tingsnotarie Lars Persson

Britt Sahlén Olfosson

Härmed intygas att Lars Persson egenhändigt vidimerat denna handling och att han därtill är behörig.
Lund den 12 augusti 1993
Ex officio:

Dnr 303/93
Avgift SEK 100

Helén Kinnman
Notarius Publicus

NOTARIUS PUBLICUS I LUND

No. 9748

The Ministry for Foreign Affairs in Stockholm hereby certifies that

Mr Lars Jersson
District Court
Lund

has issued and signed the foregoing attestation in his official capacity

Fee Kr V.g.

Stockholm this 18th day of August 19 93

Örjan Landelius

EXPEDITIONSAVGIFT
18. 8. 93 000:-

GENERAL AUTHENTICATION CERTIFICATE

KINGDOM OF SWEDEN)
CITY OF STOCKHOLM) SS
EMBASSY OF THE)
UNITED STATES OF AMERICA)

I do hereby certify that the official named below, whose true signature and official seal are, respectively, subscribed and affixed to the annexed document, was, on this day, empowered to act in the official capacity designated in the annexed document, to which faith and credit are due.

Örjan Landelius, Counsellor, Second Division, Legal Department
(Typed Name of Official) Ministry for Foreign Affairs
Stockholm, Sweden

(Signature of Consular Officer)

Justice B. Stevens
Consul of the United States
of America
(Name of Consular Officer)

August 23, 1993
(Date)

(SEAL)

DOCKETED
filed 11/24/93

förmynderskapsdomstol

LUNDS TINGSRÄTT

Rätt ns

FÖRMYNDERSKAPSBOK - god man

Den för vilken god man förordnats (namn, yrke och hemvist; för bortavarande delägare i dödsbo även den dödes namn och sista hemvist)

Torper, Anders Olof, 760128-4032, Drapavägen 69, 224 74 LUND

Förordnandet avser

att såsom god man enligt 11 kap 2 § föräldrabalken bevaka Olof Torpers rätt vid boutredning samt vid bodelning och skifte eller ingående av avtal om sammanlevnad i oskiftat dödsbo avseende dödsboet efter Anneli Marie Tallberg Torper, Drapavägen 69, LUND

God man (namn, yrke, hemvist och postadress)	Datum för förordnande / nummer	Datum för entledigande / nummer
Tallberg, Tommy, 401112-4510, Nyckelkroken 25, 226 47 LUND	1993-01-26 FM 22	

God man vid boutredning samt vid bodelning och skifte eller ingående av avtal om sammanlevnad i oskiftat dödsbo/vid mottagande av gåva

Boupptecknr	Registernamn	Kommun
	Torper, Ulf, barn	LUND

DV 594 A 1992 Nyköpings AB Bjärnum

LUNDS TINGSRÄTT
☐ Avskriften ☒ Kopian
överensstämmer med originalet

Lars Persson
tingsnotarie

No. 9352
The Ministry for Foreign Affairs in Stockholm hereby certifies that
Mr. Lars Persson
District Court
Lund
has issued and signed the foregoing attestation in his official capacity

Fee Stockholm this 18th day of August
Kr. 100:- 19 93

Örjan Landelius

EXPEDITIONSAVGIFT 000:-

Härmed intygas att Lars Persson egenhändigt vidimerat denna handling och att han därtill är behörig.
Lund den 12 augusti 1993
Ex officio:

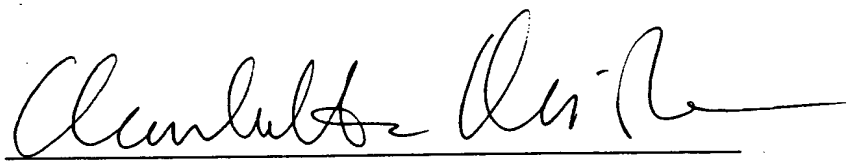
Dnr 303/93
Avgift SEK 100

Helén Kinnman
Notarius Publicus

NOTARIUS PUBLICUS I LUND

I, the undersigned Charlotta Kristensson, Bachelor of Arts, of Bellevuevägen 46, Malmö, Sweden, do hereby declare that I am conversant with the English and Swedish languages and a competent translator thereof, and I further declare that to the best of my knowledge and belief the following is a true and complete translation made by me of the annexed document.

Malmö, this 11th day of November 1993



Charlotta Kristensson

Law Offices of
HEDENER & PENSER
ADVOKAT LENNART HANSSON AB
ESLÖV

E S T A T E I N V E N T O R Y

Starting on 13th August 1992, an inventory has been made of the property left by Anneli Marie Tallberg Torper, Drapavägen 69, S-224 74 Lund, born on 15th September 1945 and deceased on 11th July 1992.

The deceased leaves as parties to the estate:

HER CHILDREN

1. Maria Alice Torper, 740223-3923, Drapavägen 69, S-224 74 LUND
2. Anders Olof Torper, 760128-4032, Drapavägen 69, S-224 74 LUND

It was noted that the following persons were present at the estate inventory proceedings: co-habitant Eskil Persson, Drapavägen 69, S-224 74 Lund, now Kastanjegatan 21:7, S-224 56 LUND, beneficiary Ulf Torper, Sångarevägen 20 E, S-224 71 LUND, as guardian of Olof Torper, and Tommy Tallberg, 401112-4510, Nyckelkroken 25, S-226 47 LUND, as proposed legal representative of Olof Torper.

It was noted that the deceased leaves a will of 25th June 1992, Encl. 1.

It was further noted that Eskil Persson has called for a division of the joint property in view of the cohabitation, Encl. 2.

The estate was accounted for by Eskil Persson. The assets and the debts were noted and evaluated as follows.

Law Offices of
HEDENER & PENSER
ADVOKAT LENNART HANSSON AB
ESLÖV

W I L L

I, Anneli Maria Torper-Tallberg, born on 15th September 1945, do hereby declare this to be my last will and testament in the manner and form following.

1. Volvo NTB 873 to Ulf Torper, 450727-....

2. 2 checked sofas to Eskil Persson, 300416-4152

3. 2 Lundquist bookshelves to Eskil Persson, 300416-4152

4. 1 Denon CD player to Eskil Persson, 300416-4152

5. 1 Tibetan mat, dragon subject, to Maria Torper 740223-3923

6. 1 doctor's ring to Olof Torper, 760128-4032

Lund, this 25th day of June 1992

Signature
Anneli Torper-Tallberg

That Anneli Torper-Tallberg, whom we know in

person, has this day, of sound mind and body and of her own free will, in our joint presence declared the above provisions as being her last will and hereinunder signed her name, is hereby certified by the undersigned as attesting witnesses.

Lund as stated above

Signature

Name: Bo Persson

Profession: Registered doctor

Address: Vegagatan 5
S-224 17 LUND

Signature

Name: Georges Guedj

Profession: Nurse

Address: Kollegiev. 134
S-224 73 LUND

Guardianship Court
LUND DISTRICT COURT

GUARDIANSHIP BOOK
of the Court - legal representative

He for whom a legal representative has been appointed (name, profession and residence: for absent party to the estate also the name and last residence of the deceased)

Torper, Anders Olof, 760128-4032, Drapavägen 69, S-224 74 LUND

The appointment concerns

to look after, as legal representative under the Children and Parents Code, Chapter 11, Sec. 2, the right of Olof Torper in the administration of the estate and in the division of the joint property and the distribution thereof or the making of an agreement on undivided possession of the estate regarding the estate of Anneli Marie Tallberg Torper, Drapavägen 69, LUND

Legal representative (name, profession, residence and postal address)	Date of the appointment	No.	Date of dismissal	No.
Tallberg, Tommy, 401112-4510, Nyckelkroken 25, S-226 47 LUND	26 January 1993	FM 22		
Legal representative in the administration of the estate and in the division of joint property and the distribution thereof or the making of an agreement on undivided possession of the estate/upon reception of a gift	Signature			

Estate Inventory No.	Name of Register	Municipality
	Torper, Ulf, child	LUND

LUND DISTRICT COURT
[x] This is a true copy
of the original

Signature
Lars Persson
Clerk of the District Court